Role of T cell genotype in the development of allogeneic responses after stem cell transplantation and cellular immunotherapy

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Aan Birgit & Iris

" … I think everybody has got to realise this, that we are confined by our conceptual framework all the time, even though we do not know it".
-Kevin Lafferty, *Horizon* (1997)

"Bravely I look further than I see".
-Sarah & Gert Bettens, *Believe* (1998)

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CHAPTER 1

Introduction

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1. Allogeneic hematopoietic stem cell transplantation

TRANSPLANTATION OF ALLOGENEIC HEMATOPOIETIC STEM CELLS (SCT) following high-dose systemic chemotherapy or chemoradiotherapy is the most effective curative treatment for patients with leukemia or other hematological malignancies. The pre-transplant conditioning regimen induces a major cytoreduction of malignant hematopoietic cells. However, it also results in the destruction of the bone marrow and normal hematopoietic cells. The recipient's hematopoietic system is restored by infusion of hematopoietic stem cells from a donor, which are capable of reconstituting sustained hematologic and immunologic function. Most hematological malignancies exhibit a steep dose-response reaction to chemoradiotherapy. However, not every malignant cell may be killed, even when the conditioning regimen has been intensified to levels at which serious organ toxicities are encountered[1,2].

It is now clear that an additional component contributes to the antileukemic properties of allogeneic SCT. This component is associated with immunocompetent cells transplanted with, or arising from, the donor graft, and is termed graft-versus-leukemia (GVL) reactivity.

1.1. GVL reactivity and graft-versus-host-disease

The earliest evidence that a potent GVL reactivity is associated with allogeneic SCT was found in murine transplant experiments. Leukemic mice treated with radiation therapy died of recurrent leukemia if transplanted with syngeneic marrow, whereas mice transplanted with allogeneic marrow did not relapse[3]. The recipients of allogeneic marrow, however, died of graft-versus-host disease (GVHD), an immunological response directed towards antigens expressed by normal host tissues, demonstrating that GVL reactivity and the development of GVHD are intimately associated processes.

Most evidence for GVL reactivity is based on several indirect clinical observations. Recipients of marrow grafts from identical twins, and recipients of autologous grafts, are more likely to develop a relapse than recipients of matched sibling grafts[4]. Furthermore, the occurrence of GVHD after allogeneic BMT is associated with a lower risk of relapse[5-7]. Moreover, some recipients of allogeneic SCT achieved complete remissions of relapsed leukemia or leukemia refractory for conventional chemotherapy, after a flare of GVHD, or following withdrawal of immunosuppressive therapy, suggesting that immunocompetent cells are involved[8-10].

1.2. Donor-derived T cells mediate anti-host reactivity

The development of GVHD is a major complication of allogeneic SCT. The incidence and severity of GVHD can be reduced by depletion of T lymphocytes from the stem cell graft. T cell depletion, however, is associated with an increased risk of leukemia recurrence[5,11]. Furthermore, *in vivo* primed anti-host donor-derived cytotoxic T lymphocytes (CTL) can be readily obtained from the peripheral blood of recipients of allogeneic BMT[12-18]. Some of these CTLs effectively lyse leukemic cell precursors and circulating myeloid and lymphoid leukemia cells, but not cells derived from GVHD target organs such as skin fibroblasts, keratinocytes, or liver cells[16,18-22]. Direct clinical evidence for T cell-mediated GVL reactivity has been provided by the transfer of donor peripheral blood mononuclear cells (PBMC), containing large numbers of T cells, to treat patients with relapsed leukemia[23-27]. These T cell infusions induced both complete cytogenetic remissions and the development of GVHD. These observations re-affirm the intimate association of GVHD with GVL reactivity and demonstrate that donor-derived T cells play a key role in mediating anti-host immune reactivity.

Clinically significant acute GVHD develops in 9-50% of patients who receive a human leukocyte antigen (HLA)-identical graft[28,29]. This suggests that incompatibilities for non-major histocompatibility complex (MHC)-encoded minor histocompatibility antigens (mHags) play an important role in the induction of T cell mediated alloimmune responses.

1.3. Minor histocompatibility antigen incompatibilities induce T cell responses after HLA-matched allogeneic SCT

Minor Hags are peptides that are derived from proteins that differ between the recipient and donor due to polymorphisms in the genome. The polymorphisms that give rise to mHags encode changes in amino acid sequence that result in altered binding of peptides to the MHC, or altered contact between the MHC/peptide-complex and the T cell receptor (TcR), or differential processing of the polymorphic cellular protein[30] (Table 1). For example, the change in amino acid sequence of mHag HA-1 causes the non-immunogenic allelic peptide not to be expressed at the cell surface by HLA-A*0201 molecules[31]. A proline to arginine substitution in the HA-8 mHag causes differential processing of the polymorphic peptide, which facilitates the transport of the immunogenic allelic variant of the HA-8 peptide into the endoplasmatic reticulum by TAP (transporter associated with antigen processing)[32]. Another example of differential processing of polymorphic peptides is the differential cleavage of allelic variant peptides of mHag H-Y by the proteosome, resulting in premature destruction of the CTL epitope. In summary, due to genetic differences outside the MHC complex, the repertoire of endogenous peptides displayed in the peptide-binding groove of HLA-matched siblings may differ substantially. These incompatibilities in displayed peptide repertoire may constitute immunogenetic differences that induce (reciprocal) allogeneic T cell responses.

Most mHags have a broad tissue expression. Therefore, mHags expressed by both hematopoietic cells as well as by epithelial cells present in skin, liver, and gut, will induce GVHD, including GVL reactivity. Minor Hag expressed exclusively by hematopoietic or leukemic cells are of particular interest, since they may elicit a selective GVL-response, and could be used as target antigens for specific immunotherapy without causing GVHD.

Several mHags have been characterized at the molecular level, with different expression patterns (*i.e.* ubiquitously, tissue restricted, or leukemia-associated), HLA-restriction, and phenotype frequency (Table 1).

Table 1. Human mHags characterized at the molecular level.

mHag	HLA-restriction	Antigenic peptide*	Tissue distribution	Effect of polymorphism on	Encoding gene	Chromosome	Refs.
HA-1	A2	VL**H**DDLLEA	Hematopoietic cells	MHC/peptide binding	*KIAA0223*	19p13	[31,38]
HA-2	A2	YIGEVLVS**V**	Hematopoietic cells	n.d.	*MYO1G*	7p13	[38]
HB-1	B44	EEKRGSL(**H/Y**)VW	B lymphoid cells	TcR recognition	*HB-1*	5q31	[40]
HA-8	A2	**R**TLDKVVLE**V**	ubiquitous	TAP processing	*KIAA0020*	9q22	[32,38,39]
H-Y	A1	IVD**C**LTEMY	ubiquitous	TcR recognition	*DFFRY*	Y	[37]
H-Y	B7	SP**S**VDKA**R**AEL	ubiquitous	TcR recognition	*SMCY*	Y	[36]
H-Y	A2	FI**D**S**Y**I**C**Q**V**	ubiquitous	n.d.	*SMCY*	Y	[35]
H-Y	B60	**R**EESEE**ES**V**S**L	ubiquitous	Proteasome cleavage	*UTY*	Y	[34]
H-Y	B8	LPHN**H**T**D**L	ubiquitous	n.d.	*UTY*	Y	[33]

n.d., not determined; * in bold: polymorphic immunogenicity-inducing amino acid(s)

1.4. Mechanism of the alloimmune response after HLA-matched hematopoietic SCT

The generation of alloimmune responses after hematopoietic SCT is a multistep process, mediated by T lymphocytes, involving different effector cells and inflammatory cytokines (Figure 1). Primary allogeneic responses are initiated when immunogenetic mHag disparities and possibly leukemia-associated antigens are presented to naïve donor T cells by activated host antigen-presenting cells (APCs)[41,42]. Host dendritic cells (DCs) play a pivotal role as professional antigen presenting cells[43].

The conditioning regimen before allogeneic SCT damages host tissues (including DCs), resulting in the release of inflammatory cytokines (*e.g.* IL-1, TNF-α, GM-CSF, and IFN-γ)[44-46]. These inflammatory cytokines upregulate adhesion molecules and MHC antigens[47,48], which may enhance the recognition of host-tissue antigens by donor-derived T cells after transplantation. In addition, the damage caused by extensive conditioning causes breakdown of mucosal barriers, such as the gastrointestinal tract, resulting in increased bacterial lypopolysaccharide (LPS) translocation[49]. This bacterial product serves as a 'danger signal' that can trigger the activation of DCs.

Activation of APCs results in an increased surface expression of MHC, co-stimulatory (CD80 (B7.1), CD86 (B7.2)) and adhesion molecules (*e.g.* CD54 (ICAM), CD11a (LFA-1)). This makes the APC ideally suited for recognition by T cells. CD4+ T cells are activated after specific binding of their TcR to a MHC class II/peptide-complex on the activated APCs (signal 1: antigen-specific stimulation) in combination with an interaction of co-stimulatory molecules and their ligands on the T cell (signal 2: co-stimulation). This physical APC-T cell interaction, referred to as the 'immunological synapse'[50], results in the production of activating (Th1) or inhibitory (Th2) cytokines by the CD4+ T cell that regulate the immune response. Furthermore, coupling of the CD40 ligand (CD40L), which is expressed on activated CD4+ T cells, to the CD40-receptor on the APC, upregulates the expression of MHC class I and co-stimulatory molecules on the APC, and the secretion of cytokines by the APC. This stimulation increases the ability of APCs to activate naïve CD8+ T cells, initiating the effector arm of the immune response. Thereafter, T cells clonally expand, while other cell types are recruited, such as macrophages, granulocytes, natural killer cells, large granular lymphocytes. The T cells and other cell types are responsible for the execution of the alloresponses associated with GVHD and GVL.

T cell effector responses are further regulated in the periphery by recently described novel members of the B7-family of costimulatory ligands, such as B7h, PD-L1, PD-L2, and B7-H3, reviewed in[51]. Unlike CD80 and CD86 that are involved in the *initiation* of adaptive immune responses, these ligands are also expressed on non-lymphoid tissues at sites of inflammation. Their receptors are induced on activated T cells, while CD28, the activating receptor for CD80 and CD86, is expressed on both activated and naïve T cells.

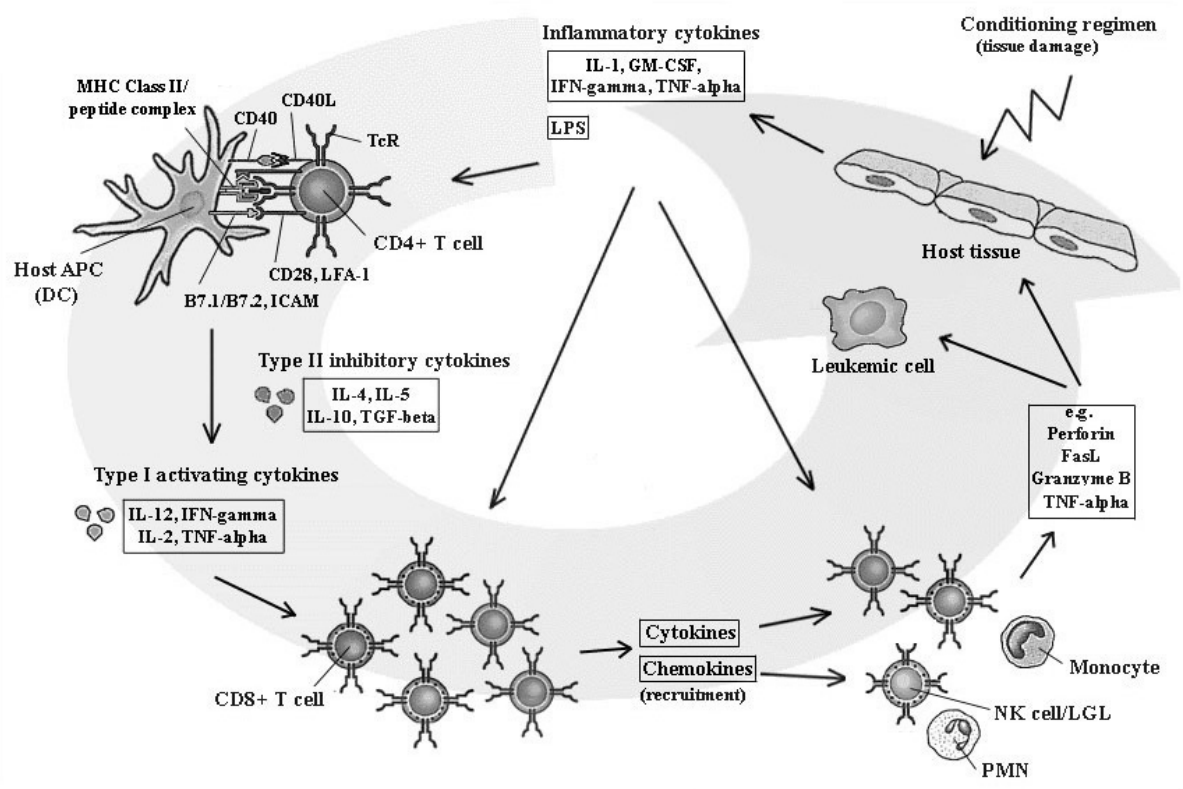

Figure 1. *Generation of an immune response after HLA-matched allogeneic HSCT. LGL, large granular lymphocyte; PMN, polymorphonuclear leukocyte.*

1.5. Variables influencing the development and intensity of an alloimmune response after SCT

Several variables associated with allogeneic SCT influence the development and intensity of an alloimmune response after SCT. These variables, the way their influence is mediated, and selected published findings, are summarized in Table 1.

The immunogenicity of the leukemic cells influences the ability of donor-derived T cells to generate a GVL response. Leukemic cells generally lack expression of co-stimulatory molecules, although leukemic-cell-derived APC have been identified and generated[52-54]. The conditioning regimen contributes to the pathogenesis of GVHD, as described above. Furthermore, it ablates host APCs, and exerts host immunosuppresion, contributing to the extent of engraftment and outgrowth of donor hematopoietic cells. The graft provides the T cells that mediate anti-host responses. Depletion of T cells from the graft and the immunogenetic features of the graft, contribute to the intensity of (reciprocal) alloresponsiveness. GVHD prophylaxis after SCT may reduce and even abrogate anti-host alloresponses. The genetic origin of the cells that constitute the hematopoietic compartment is of particular interest for this thesis, since the genetic origin of these cells, and in particular those that exert immunological function, may influence the response to immunotherapy given after allogeneic SCT (Table 1.).

Table 1. Variables influencing the development and intensity of alloimmune responses after SCT

Variable	Influence through	Selected findings and comments
Leukemic cells	Immunogenicity of the leukemia: - ability to present antigens. - presence of co-stimulatory molecules.	- Dendritic cells derived from Ph+ chromosome-positive CD34+ cells are competent APCs and can induce antileukemic T cell responses[52,53]. - Leukemic monocytes in CML are competent APC and can capture, process and present exogenous immunogenic peptides derived from leukemic cell breakdown to T cells[54].
Conditioning regimen before SCT	Intensity of conditioning regimen: - contributes to the pathogenesis of GVHD. - ablates potential players that mediate the alloimmune response (*i.e.* host APCs and T cells). - influences the extent of chimerism.	- The intensity of conditioning regimes varies to a great extent, from myeloablative, to non-myeloablative and to reduced intensity. - The high variety of conditioning regimes causes variable damage and activation of host tissues and subsequent release of inflammatory cytokines and enhancement of recipient MHC antigen expression. - Augmented release of bacterial breakdown products, such as LPS, and potential cross-reaction of donor T cells with bacterial antigens may facilitate the allorecognition process.
Graft	- Depletion of immunocompetent cells from the graft.	- Reduced incidence and severity of acute GVHD[5,61]. Development of mixed chimerism.
	- Histocompatibility of the graft.	- HLA-mismatched donor-recipient couples: a dramatic GVH reaction commonly occurs, even with a single antigen difference[62,63]. When the recipient and donor are HLA-identical, miH antigens induce alloresponses with varying degrees of severity[64-66].
	- Gender mismatching.	- increased risk of acute GVHD (especially female multiparous donor and male recipient)[63,67].
Chimerism	Genetic origin of the players mediating the alloimmune response.	- Recipient DCs stimulate donor-derived T cells[43]. - Potential reciprocal T cell responses[55,56](Chapter 4; this thesis). - Development of regulatory T cells[57-60].
GVHD prophylaxis	Dose of methotrexate and cyclosporin.	Affects the development of GVHD (reviewed in[29]).
Immunotherapy	Infusion of mediators/effectors of alloreactivity.	Modulates the alloimune response - *e.g.* therapeutic/profylactic DLI, *in vitro* generated CTLs.

1.5.1. Definition and development of hematopoietic chimerism after allogeneic SCT

The term hematopoietic chimerism refers to the presence of hematopoietic cells of non-host origin. This exceptional immunogenetic state can develop after allogeneic hematopoietic SCT. The conditioning treatment allows the engraftment and outgrowth of hematopoietic cell populations of donor origin. A mixture of donor and host hematopoietic cells is called mixed chimerism. Full or complete chimerism refers to complete replacement of host by donor hematopoiesis.

Most patients undergoing unmanipulated allogeneic hematopoietic SCT will become full hematopoietic chimeras. Presumably, T cells in the graft contribute to the establishment of full chimerism by a graft-versus-recipient-hematopoiesis response. In contrast, if the graft is T cell depleted[68-72], or if the conditioning regimen is reduced in intensity[73-79], development of mixed chimerism in patients is observed more frequently.

1.5.2. Detection of chimerism

Differences between donor and recipient in polymorphic genetic markers or their products have been used for the detection of chimerism by employing a variety of molecular methods with various sensitivities[80-89]. The most generally applied method to evaluate chimerism is restriction fragment length polymorphism (RFLP) analysis. The introduction of polymerase chain reaction (PCR) as a method for rapid amplification of minisatellite (variable number of tandem repeats; VNTR) and microsatellite (short tandem repeats; STR) sequences, has significantly enhanced the sensitivity of detection of chimerism. The fluorescent *in situ* hybridisation (FISH) technique is also widely applied for the detection of chimerism, but is restricted to sex-mismatched donor-recipient couples[90]. The value of chimerism analysis is increasingly appreciated in understanding the development of allogeneic immune responses and monitoring imminent leukemic relapse[91-93]. The success of adoptive immunotherapy may be enhanced with frequent analysis of chimerism, using sensitive and accurate quantification methods for optimal diagnosis[94-96]. Therefore, we have developed a real-time PCR-based method using single nucleotide polymorphisms (SNP) as discriminative markers (Chapter 5; this thesis)[97].

1.5.3. T cell chimerism status in regard to the development of alloimmune responses after SCT

Several clinical studies have indicated a correlation between mixed hematopoietic chimerism and a reduced incidence of acute GVHD, compared to full chimerism, after myeloablative allogeneic SCT[69,98-101]. In two of these studies, however, a significant number of patients received a T cell depleted graft[69,98], which in itself is associated with a reduced incidence of GVHD and a more common development of mixed chimerism. Furthermore, patients were given GVHD prophylaxis when deemed necessary. Mattsson *et al*. have lineage-specified the correlation between mixed hematopoietic chimerism and a reduced incidence of GVHD to mixed chimerism in the T cell population[102]. They argued that the failure of some other studies[103-105] to show this relationship could be due to low T cell numbers during the first months after SCT, and the unavailability of sensitive methods for chimerism analysis.

Few have studied the effect of T cell chimerism on the development of immunoresponses after non-myeloablative allogeneic SCT[106,107]. Conflicting results have been described. Childs *et al*. showed that acute GVHD only occurred in patients who achieved full donor T cell chimerism, while in the study of Mattsson *et al*. mixed T cell chimerism did not protect against acute GVHD. It was unclear, in the latter study, why patients with a high degree of remaining recipient T cells may still develop acute GVHD. The lack of accordance between the findings of these studies may be accounted for by the use of different conditioning and immunosuppressive regimens.

The correlation between mixed hematopoietic chimerism and relapse is extensively studied. Several studies have suggested an increased occurrence of relapse of CML in patients with mixed hematopoietic chimerism, compared to patients that have developed full hematopoietic chimerism[70]. However, other studies have failed to show the correlation between mixed chimerism and an increased risk of relapse, or failed to show this correlation for relapse of acute leukemias[69,105,108,109]. Residual recipient hematopoietic cells at best may reflect healthy hematopoiesis or residual lymphocytes that survived chemotherapy, or at worst cells derived from the malignant clone. Furthermore, mixed hematopoietic chimerism may reflect a state of immunotolerance between donor and recipient, allowing more host-type cells to survive. Mackinnon *et al*., however, have shown a correlation between mixed T cell chimerism and an increased relapse for CML, suggesting a correlation with GVL reactivity[70]. This correlation between mixed hematopoietic chimerism and reduced GVL reactivity was also suggested for other diseases, such as lymphoma[110].

Taken together, these studies show that mixed hematopoietic chimerism, and more importantly T cell chimerism (being the prime cell population modulating allo immune responses after SCT), is correlated to a reduction in the development of donor immune reactivity towards the recipient (*i.e*. GVHD and possibly GVL reactivity), compared to full chimerism. Still, GVHD can develop and disease response can occur. Furthermore, these studies show the role of multiple variables in the development of immune responses after SCT, and demonstrate how these variables influence each other (Figure 2).

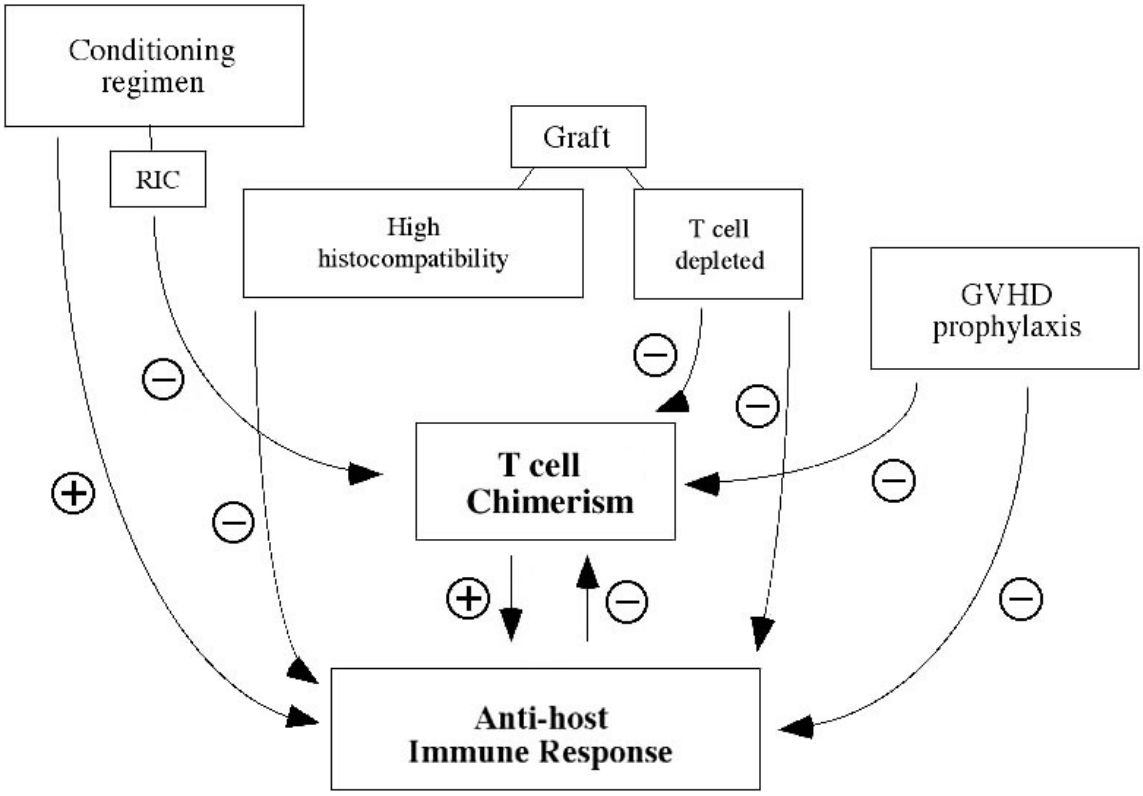

Figure 2. *Interaction of variables associated with SCT that influence the development and intensity of anti-host immune responses after allogeneic SCT. Increased magnitude of respective variables either increase (⊕) or reduce (⊖) T cell chimerism or anti-host cellular immunity. The latter variables interact with each other.*

2. Adoptive immunotherapy after allogeneic SCT

Clear clinical evidence shows that immune-mediated elimination of leukemia contributes to the success of hematopoietic SCT. Immune-based therapies such as antibody and cytokine therapy have already been successfully developed and incorporated into standard treatment regimens for some human malignancies. Cellular immunotherapy is an attractive approach to augment GVL reactivity after allogeneic SCT to prevent or treat relapse. However, alloresponses of adoptively transferred unselected leukocytes are initiated towards a wide range of alloantigens. The development of methods to activate and expand effector cells with defined specificity and function should improve the effectiveness and safety of this form of therapy.

2.1. Donor leukocyte infusions to treat relapse after allogeneic SCT.

The infusion of leukocytes from the original donor, administered separately from the initial transplant, to treat relapsed CML was first described a decade ago[23,24]. These donor leukocyte infusions (DLI), containing large amounts of T cells, induced complete cytogenetic remissions and provided the first direct clinical evidence for GVL reactivity.

DLI have been especially successful in the treatment of relapsed CML in chronic phase, where complete remissions have been reported in about 80% of patients. Patients in more advanced stages of CML, and with acute leukemia, obtain a lower

percentage of response (*i.e.* 12-28% for advanced phase CML, 22% for AML and 8% for ALL)[25-27].

Acute and chronic GVHD develops in approximately 60% of patients after DLI, and has contributed to death in almost 10% of patients who develop GVHD[25]. Several approaches have been developed to reduce GVHD after DLI. In small studies, the infusion of escalating doses of T cells[111,112], the introduction of suicide genes into transferred cells[113-117], or the administration of T cells after the selective depletion of $CD8^+$-subsets[118,119], resulted in a reduced incidence and/or severity of GVHD. These approaches may be beneficial for patients with CML.

To control GVHD after T cell depleted allogeneic SCT DLI has been successfully applied as a delayed add-back of T cells[120-126]. Several mechanisms may contribute to a decrease in the development of GVHD after delayed infusion of donor lymphocytes, including the avoidance of the 'cytokine storm' induced by pretransplant conditioning[46,127,128], the presence of fewer host-type APCs[43], and the development of immunosuppresive regulatory cells[57,129]. In addition, T cell depletion itself reduces GVHD. GVL reactivity was preserved as shown by salvage of 72-89% of relapses[120-123] and favourable relapse-rates in patients who are at high risk for relapse[126]. Caution should be taken, however, since T cell depletion increases the risk of fatal graft rejection, and even if recovery of autologous hematopoiesis ensues, the patient has been sensitized to the donor and may reject subsequent DLI.

DLI-induced pancytopenia is somewhat less common than GVHD after DLI, occuring in 18-50% of patients[130,131]. Aplasia resulting from DLI to treat patients that developed a full clinical relapse, in general, is mild and transient[132].

2.2. DLI after non-myeloablative allogeneic stem cell therapy

High-dose chemoradiotherapy may not completely eradicate all host normal and malignant hematopoietic cells, even after intensification of the pretransplant therapy to a point where non-marrow organ toxicities become limiting[1,2]. DLI can induce complete remissions in patients who relapsed with leukemia after hematopoietic SCT, even when the leukemic cells are resistent to chemoradiotherapy. This has led in recent years to a conceptual shift in allogeneic hematopoietic SCT from trying to eradicate malignant cells through maximally tolerated doses of toxic therapy, towards using allogeneic leukocytes to eliminate the malignancy. Alternative conditioning regimens with reduced toxicity are being developed, which allow initial engraftment of donor effector cells (*i.e.* induce HVG tolerance), with these cells being the primary therapeutic modality[73-78]. DLI in this setting is intended to effect a powerful lymphohematopoietic GVH-reaction to consolidate donor engraftment and enhance GVL reactivity. However, the optimal usage has yet to be determined.

Major responses have been seen in a variety of hematologic malignancies, primarily including patients with highly chemorefractory disease[73-78]. GVHD remains a major clinical concern and treatment challenge. Follow-up data are limited and additional time is needed to determine the efficacy and toxicities of this form of immunotherapy.

2.3. T cell chimerism status in regard to the development of DLI-induced alloimmune responses

The mixed T cell chimeric state represents a co-existence between immune effector cells that may exert potential reciprocal reactivity. Following non-myeloablative conditioning and in situations in which host hematopoiesis has returned with relapse of hemological malignancy, host T cells can be present in substantial numbers, and consequently influence the development of DLI-induced alloimmune responses. Several groups have described the role of regulatory cells in the development of immune tolerance after BMT[57,133,134]. Johnson *et al*. identified a donor-derived immunoregulatory CD4+/CD25+ T cell population that suppresses GVH reactivity in a MHC-mismatched murine model for DLI[57,129]. A clinical study by Schattenberg *et al*. showed that the percentage of donor-derived T cells at the time of DLI significantly correlated with the occurrence of GVHD and induction of remission[55]. Patients treated with DLI have immunocompetent T cells. If these cells are of recipient origin they may exert an alloresponse towards infused donor lymphocytes and subsequently cause rejection. Blazar *et al*. showed that host T cells are capable of generating anti-donor CTL reactivity that results in an impaired ability of DLI to induce GVHD in murine models[56]. We have shown that a predominance of recipient T cells at the time of DLI significantly reduces the survival time of infused donor T cells in a rat model (Chapter 4; this thesis). This fast disappearance suggests that this elimination of donor T cells is an active process possibly mediated by alloreactive sensitized recipient T cells. Moreover, Spitzer *et al*. found that donor T cell levels ≤20% were associated with graft loss after non-myeloablative allogeneic SCT, despite subsequent DLI, suggesting a strong HVG response[73]. Keil *et al*., however, described a patient who had full recipient hematopoiesis at the time of DLI to develop aplasia, resulting from a DLI-induced T cell response towards the hematopoietic compartment[132].

2.4. *In vitro* generated antigen-specific donor-derived T cell clones

The adoptive transfer of T cells that are selected for specific recognition of mHags exclusively expressed by recipient leukemic cells or recipient hematopoietic cells represents a potential approach for eradicating leukemic cells without inducing GVHD. Furthermore, in this way a more potent antitumor effect can be achieved compared to unselected polyclonal donor lymphocytes used for DLI. Falkenburg *et al*. have treated a patient in accelerated phase of CML with leukemia-reactive CTL, resulting in complete molecular remission[135]. No GVHD was observed in this study. An increasing number of mHags is being characterized at the molecular level, which may broaden the application of mHag-antigen specific T cells for adoptive immunotherapy[136].

Potential targets for immunotherapy that would not require allogeneic SCT are being evaluated. These proteins include leukemia-specific proteins (*e.g*. Bcr-Abl fusion protein, PML/RARα fusion protein, and ETV6-AML1)[137-139] and leukemia-associated normal proteins (*e.g*. proteinase-3, WT-1, hdm2, and hTERT)[96,140-142]. Epstein Barr virus (EBV)-specific donor-derived T cell lines have been generated and infused to successfully treat EBV-induced post-transplant lymphoma and relapsed Hodgkin's disease[143,144]. Complete eradication of EBV-induced lymphoproliferative disease after DLI had been described previously[145].

Taken together, these studies have demonstrated that it is feasible to generate T cells of desired specificity *in vitro* that retain function and the ability to persist and migrate *in vivo* after infusion into patients.

3. Monitoring T cells after adoptive therapy

The ability to study the fate of infused cells used for adoptive immunotherapy can provide valuable biological information about the mechanisms involved in the development of immunological responses. Better understanding of these mechanisms may lead to the development of methods to improve the outcome of this form of therapy.

3.1. T cell marking

The addition of a marker enables to monitor and quantify the survival of infused T cells. In this way infused cells can be distinguished from pre-existing cells in the recipient. Marked T cells can be tracked to learn if they can home to specific sites and if they are involved in GVHD and GVL reactivity.

3.2. Use of retroviral vector for gene-marking of adoptively transferred T cells

Gene-marking provides a tool to mark cells not only for their entire lifespan, but also for the life span of their progeny. The majority of marker studies performed have used murine retroviral vectors to introduce foreign DNA (*i.e.* viral and transgene) sequences into a host cell. The stable introduction of a marker provides a strong advantage over the use of radioisotopes or fluorescent dyes in labeling procedures of cells. Isotope half-life, leakage of dye out of cells, and reduction of label in dividing cells will hamper accurate quantification and significantly decrease the follow-up time after transfer. Several marking studies have been performed. Table 2 summarizes the results on persistence and homing of retrovirally transduced T cells used in clinical studies. These studies show the feasibility of follow-up of infused cells, and provide valuable information about homing and accumulation of T cells at target sites. Furthermore, information on T cell persistence and dynamics, such as expansion upon Ag-encounter, and T cell eradication upon suicide induction, can be obtained.

Marked T cells can be identified and quantified via PCR of transduced DNA sequences, or via flow cytometric analysis after antibody staining of expressed retroviral transgene products.

Table 2. Homing and persistence of retrovirally transduced T cells used in clinical studies

Target T cell	Disease	Findings	Refs.
TILs	Melanoma	- Short-term detection of marked cells.	151
Cytotoxic EBV-specific	EBV-LPD	- Detection of marked cells up to 6 years post transfer. - Expansion upon EBV reactivation. - Accumulation of marked cells at disease sites.	115,143,144, 153
Cytotoxic EBV-specific	EBV genome-positive M. Hodgkin	- Detection of marked cells up to 6 months post transfer. - Accumulation of marked cells at disease sites.	152
TILs	Melanoma, renal cell cancer	- Detection of marked cells up to 9 months post transfer. - No evidence of homing.	149-151
TILs and PBLs	Melanoma, renal cell cancer	- Detection of marked cells up to 4 months post transfer. - No evidence of specific homing TILs compared to PBL.	148
Gag-specific CD8 clones	HIV-infection	- Eradication of marked cells containing Tk or hygromycin.	147
CD4+ from syngeneic twins	HIV-infection	- Trafficking of *neo*-marked cells to HIV-infected lymph nodes.	146
PBMC from HSC donor	GVH	- Detection (albeit nonquantifiable) for more than 7 months post transfer. - Eradication of Tk marked cells (complete/partial response to GCV).	113-117

TIL, tumor-infiltrating lymphocyte; EBV, Epstein-Barr virus; LPD, lymphoproliferative disease; PBL, peripheral blood lymphocyte; HIV, human immunodeficiency virus; PBMC, peripheral blood mononuclear cells; Tk, thymidine kinase; GCV, ganciclovir.

3.3. Method of retroviral T cell marking

Currently, mammalian C-type retroviral murine leukemia virus (*i.e.* Moloney murine leukemia virus (MoMLV)) based vectors are the predominant vehicles for gene gene-delivery systems, especially in gene-marking studies. Retroviruses are RNA viruses that replicate via DNA proviral intermediates that can stably integrate into host DNA. Retroviral genomes are composed of *cis* elements, which are non-coding areas of the genome necessary for replication, and *trans* elements, which are coding elements that give rise to proteins (Figure 3A).

Retroviral vectors are plasmids containing recombinant proviral DNA molecules in which the packaging signal and long terminal repeat are retained (*cis*-elements), and the structural and replicative genes (*gag*, *pol*, and *env*; *trans*-elements) of a murine retrovirus are replaced by one or more genes of interest, driven by either the retroviral promoter in the 5' LTR, or by an added internal promoter (*e.g.* SV40) (Figure 3B). These plasmids are transfected or transduced into a packaging cell-line.

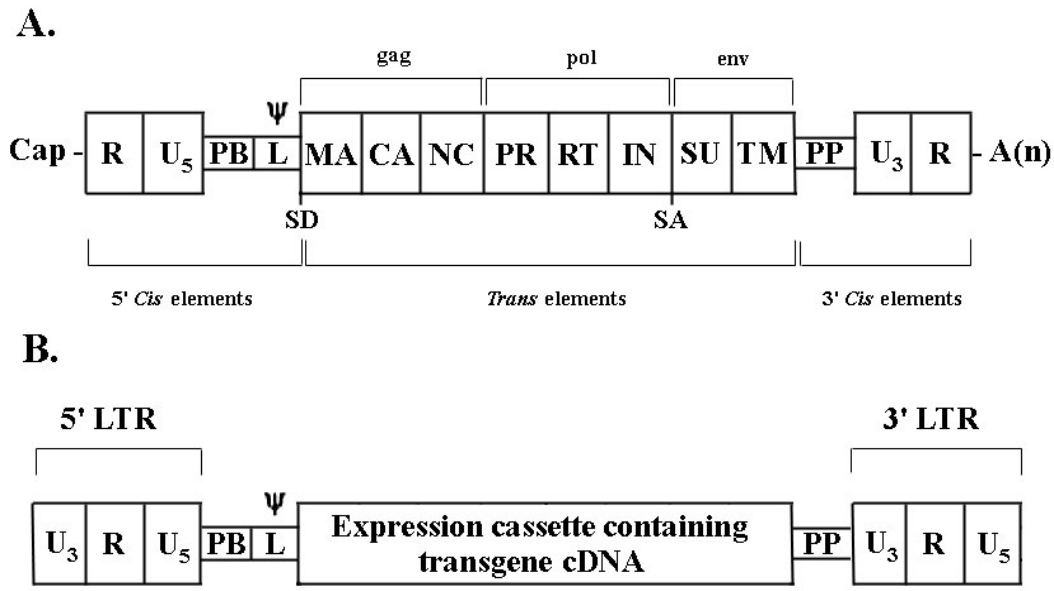

Figure 3. *Schematic map of (A) a MoMLV retroviral RNA strand (with the genome being 'diploid'), and (B) the retroviral DNA sequence of the retroviral vector. R, direct repeat; U_5, unique non-coding region forming the 3' end of the provirus genome; PB, primer binding site; L, leader sequence; ψ, packaging signal; SD, splice donor site; MA, encodes matrix protein; CA, encodes capsid protein; NC, encodes nucleocapsid protein; PR, encodes protease protein; RT, encodes reverse transcriptase protein; IN, encodes integrase protein; SA, splice acceptor site; SU, encodes surface glycoprotein; TM, encodes transmembrane protein; PP, polypurine tract; U_3, unique non-coding region forming the 5' end of the provirus genome.*

A packaging cell-line contains helper retrovirus that supplies proteins encoded by the deleted trans-elements, but generates no functional/infectious virus particles by itself (Figure 4). Transcripts from the retroviral vector, however, can be packaged by the helper virus proteins to form infectious virus. These viruses are helper free and

therefore replication-defective. They can only undergo the first stages of the virus life cycle, *i.e.* binding to the target cell, cell entry, reverse transcription into proviral DNA, and stable integration into the host genome. In general, a type C retrovirus cannot cross an intact nuclear membrane. During mitosis the nuclear membrane breaks down, allowing the reverse transcribed provirus to stably integrate into the host genome. Therefore, cells must divide to allow retroviral integration into the genome.

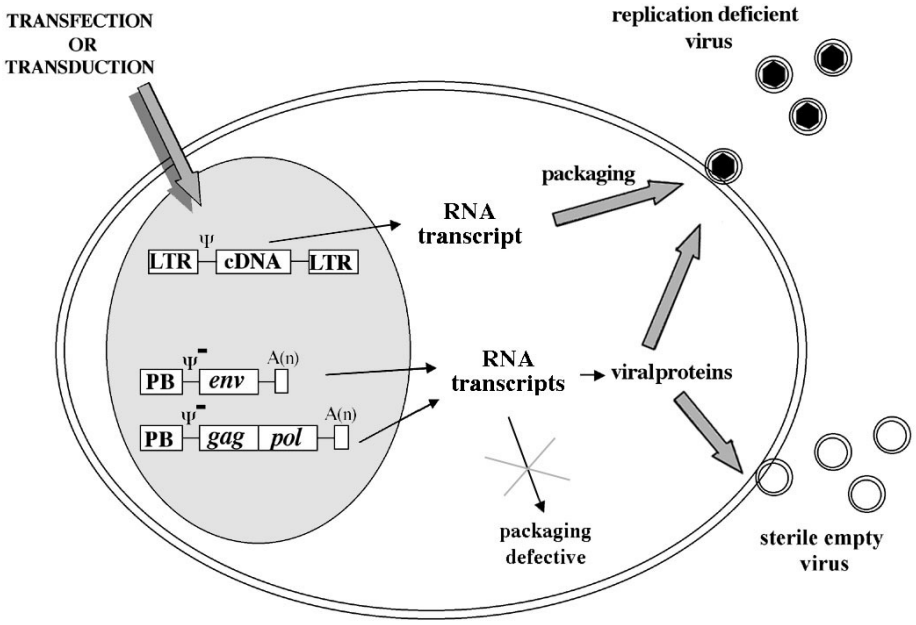

Figure 4. *Packaging cell line. Generation of replication deficient virus particles.*

The fact that T cells have to divide presents a potential drawback of this approach. *In vitro* stimulation induces phenotypic changes that may influence the survival and trafficking patterns of T cells after infusion. The potential induction of immune responses towards transduced cells is another drawback in the use of the retroviral vector system. Immunogenicity of the transduced cells is associated with the transgene products transcribed from the retroviral vector. For some gene products, such as the hygromycin-thymidine kinase fusion protein, this has resulted in rapid elimination of large numbers of transduced cells in fewer than 48 hours[154]. In contrast, *neo*-positive cells *e.g.* can be detected for up to 9 years after infusion. Moreover, *neo*-marked T cells can be readily expanded in vivo by appropriate antigenic stimulation[143,153]. To prevent immunoreactivity towards gene-marked cells a non-expressed sequence is the most ideal marker. However, this excludes the posibility to select and purify transduced cells via antibody selection or drug-resistence.

The advantages and disadvantages of the use of the retroviral vector system for T cell marking studies are summarized in Table 3.

Table 3. Advantages and disadvantages of the murine retroviral vector system for T cell marking studies

Advantages:
- Stable integration into the host genome.
- Minimal/controllable immunogenicity.
- Stable packaging system.
- Broad host range[155].
- Extensively used[156].
Disadvantages:
- Integration restricted to dividing cells[157].
- Safety issues (*i.e.* insertional mutagenesis, replication-competent retrovirus, contaminants in retroviral vector)[158].
- Sometimes limited transduction efficiencies.

4. Scope of this thesis

The scope of this thesis encompasses the role of T cell genotype (dynamics) in the development of alloreactive immune responses and the means to investigate these processes.

Chapter 2 describes a sensitive method for the *in vivo* detection and quantification of infused T cells. Genetic marking of these cells allows them to be discerned from pre-existing cells in the recipient. The method allows accurate quantification of marked cells and enables long-term *in vivo* detection of labeled cells and their progeny in peripheral blood and tissues. *In vitro* stimulation of T cells is a prerequisite for retroviral T cell marking. Also, many adoptive cellular (immuno)therapeutical approaches involve the use of *in vitro* manipulated (T) cells. We studied the potential consequences of prolonged *in vitro* culturing of T cells before infusion on the *in vivo* distribution and retention of these cells in a mouse model.

In Chapter 3 we established the specific strenghts of the real-time quantitative PCR methodology for studying the biodistribution and retention time of cultured/retrovirally labeled cells *in vivo*, in comparison to other quantitative methodologies. Furthermore, we extended its applicability, when we used it in a rat BMT model. We studied the involvement of HSV-Tk transduced T cells in the development of GVHD-induced lesions and provided a platform to monitor their persistence after ganciclovir induced suicide.

Following non-myeloablative conditioning and in situations in which host hematopoiesis has returned with relapse of a hematological malignancy, host T cells can be present in substantial numbers. We hypothesized that T cells of recipient origin, present at the time of DLI, may inhibit alloreactivity of infused donor

lymphocytes, and that this inhibition possibly is induced via elimination of infused donor cells. This might seem predictable. However, host T cells that have co-existed with donor BM-derived cells could have become tolerant to donor alloantigens[56]. In Chapter 4 we studied the *in vivo* survival of infused donor T cells in a rat model, using the method described in Chapters 2 and 3. Rats were given different treatments before DLI, thus influencing the dynamics of immunogenetic reconstitution. Consequently the survival of infused donor T lymphocytes was affected.

Several methods for the detection of chimerism have been published[80-88]. Each technique has advantages and disadvantages demonstrated by the great diversity of assays and constant development of new detection methods. The majority of these assays allow only qualitative or semi-quantitative information on the degree of chimerism. Highly sensitive and quantitative detection methods are of special importance[102,159]. The introduction of real-time PCR allows for greater sensitivity and more accurate quantitation. Chapter 5 describes the introduction of a real-time PCR to monitor the genetic origin of hematopoietic cells, including immune effector cells and leukemic cells. In a retrospective detailed analysis of the genetic origin of lymphocytes and myeloid cells of a patient with CML who relapsed after allogeneic SCT and was given subsequent DLI, the feasibility of this powerful technique was demonstrated.

In Chapter 6, we show that the addition of idarubicine, a chemotherapeutical drug, in the conditioning regimen of patients with CML (CP1), who are transplanted with partially T cell depleted stem cell grafts from HLA-identical siblings, resulted in a significantly lower 5-year probability of relapse. Furthermore, the addition of idarubicine correlated with a significant increase in the development of clinically mild acute GVHD. Using the technique described in Chapter 5, we investigated the dynamics of immunogenetic reconstitution of highly purified leukocyte subsets. Here, we demonstrated a correlation between T cell genotype and the development of alloimmune responses.

Finally, Chapter 7 summarizes the thesis and provides general conclusions.

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CHAPTER 2

Biodistribution and retention time of retrovirally labeled T lymphocytes is strongly influenced by the culture period before infusion

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SUMMARY

T lymphocytes used for adoptive immunotherapy are often cultured before transfer to generate sufficient amounts of effector cells with desired specificity. *In vitro* activation and expansion may modify lymphocytes and consequently alter their survival and trafficking patterns after transfer and influence their potential effector capacity. In this report, the authors show that the culture period of T cells after ConA/IL-2 stimulation strongly influences the retention and tissue distribution of these cells after infusion into syngeneic C57BL/6 mice. Infused labeled cells that have been cultured for 3 days remained in the peripheral blood and organs in at least a tenfold higher number than cells cultured for 8 days. In addition, cells cultured for 3 days preferentially migrate to lungs and liver shortly after infusion, and subsequently to lymph nodes and spleen. Cells cultured for 8 days preferentially migrate to liver and can be hardly detected in lymph nodes. In contrast, labeled cells cultured for 3 days are predominantly present in lymph nodes starting from day 8 until day 28. We showed that accurate monitoring of transferred cells is feasible, which may contribute to understanding response to adoptive immunotherapy.

INTRODUCTION

ALLOGENEIC STEM CELL TRANSPLANTATION (SCT) is a frequently used form of immunotherapy for the treatment of patients with leukemia and lymphoma. Infusion of donor lymphocytes (DLI) induces clinical remission in about 80% of patients with chronic myeloid leukemia (CML) and in about 15-30% of patients with acute leukemia who relapse after SCT[1-3]. In addition, SCT in combination with DLI has shown promising potential to cure some metastatic solid tumors[4,5]. Beside treatment with alloreactive effector cells derived directly from blood and bone marrow, adoptive immunotherapy with *in vitro* generated tumor-reactive effector cells is explored. Falkenburg *et al*.[6] have treated a patient in accelerated phase of CML with leukemia-reactive cytotoxic T lymphocytes (CTL), resulting in complete molecular remission without any signs of graft-versus-host disease (GVHD). EBV-induced lymphoproliferative disease and cytomegalovirus reactivation post SCT have been frequently treated by infusion of donor-derived virus-specific CTL[7-10]. Moreover, CTL specific for hemopoietic cell-restricted minor histocompatibility antigens have been generated *in vitro*, which can be used to treat patients with leukemia who relapse after SCT[11-13].

Alloreactive effector cells given by SCT and DLI are directly transferred from donors to patients. In contrast, tumor-reactive and virus-specific CTL have been cultured to induce desired specificity and to generate sufficient amounts for effective treatment. Donor lymphocytes have been transduced *ex vivo* with the herpes simplex virus thymidine kinase (HSV-tk) suicide gene to efficiently control DLI-associated GVHD[14-16]. However, survival and trafficking patterns of T cells after infusion may be influenced by phenotypic changes of these lymphocytes induced by *in vitro* stimulation and expansion. T cells may become more susceptible to apoptosis that may influence their survival and preference of tissue distribution. Furthermore, expression of adhesion molecules and chemokine receptors on the cell surface and their activation status may increase or decrease upon activation and prolonged culturing of T cells[17]. These alterations may strongly influence the effector potential of cells infused for adoptive immunotherapy.

Here, we studied the effect of the culture period of T cells before adoptive transfer on their survival and tissue distribution. We cultured splenocytes for 3 and 8 days after ConA/IL-2 stimulation. Cells were labeled via retroviral transduction with a Moloney Murine Leukemia virus (MoMLV)-based vector to track T cells *in vivo*. We determined the percentage of infused T cells in blood and several tissues after injection into the tail vein of syngeneic mice. A sensitive real-time PCR was developed for detection and quantification of the labeled cells, which enables us to monitor very low percentages of infused cells, and the persistence of these cells in various organs.

We observed a remarkable quantitative difference of labeled T cells retrieved from blood and organs after infusion related to culture period. After transfer, cells exposed to a shorter culture period *ex vivo* survive in higher amount than T cells cultured for 8 days. Moreover, T cells cultured for different periods showed distinct patterns of tissue distribution. T cells cultured for 3 days showed a relative preferential migration to lungs within 4 hours and a subsequent homing and migration to lymph nodes and spleen. In contrast, cells cultured for 8 days showed a relative preferential migration to the liver and lungs and disappearance from all organs within 28 days. These clear differences in survival and tissue distribution between short period cultured cells (3 days) and longer period cultured cells (8 days) suggest that the *ex vivo* culture period before infusion may affect the potential number of cells that can exert effector function *in vivo*.

MATERIALS AND METHODS

Mice

Eight-week-old male C57BL/6 mice were purchased from Charles River Wiga (Sulzfeld, Germany). Mice were kept under specific pathogen-free conditions in positive pressure cabinets at the animal facilities of the University of Nijmegen and fed irradiated food and acidified drinking water.

Production of retroviral vector

The replication deficient amphotropic vector SFCM-2-producing cell line (PA317) was kindly provided by Dr. C. Bonini (Istituto Scientifico H. S. Raffaelle, Milan, Italy). This MoMLV-based vector contains the truncated form of the human low-affinity nerve growth factor receptor (ΔLNGFR) as marker gene. The virus-producing PA317 cell line was cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD) supplemented with 10% FCS, 50 U/mL penicillin, and 50 μg/mL streptomycin. When cells reached subconfluency the medium was changed for IMDM (Gibco BRL) supplemented with 10% FCS, 5×10^{-5} mmol/mL 2-mercapthoethanol, 50 U/mL penicillin, and 50 μg/mL streptomycin. Virus containing supernatant was collected after 24 hours of culturing. The supernatant was filtered (0.45 μm), snap-frozen in liquid nitrogen, and stored at -80°C. Presence of amphotropic helper virus in the supernatant was excluded by a negative result of PCR specific for 4070A amphotropic murine leukemia virus envelope sequence (Accession: M33469) in DNA of transduced splenic T lymphoblasts.

Retroviral transduction of cells

Spleens were homogenized in a filter chamber and subsequently lymphocytes were isolated by density gradient separation using Lympholyte-M (Cedarlane Laboratories Ltd., Hornby, Canada). Splenocytes (10^6/mL) were activated for 24 hours with 5 μg/mL concanavaline A (ConA; Boehringer Mannheim, Mannheim, Germany) and 300 IU/mL recombinant human IL-2 (Glaxo, Geneva, Switserland) in IMDM supplemented with 10% FCS, 5×10^{-5} mmol/mL 2-mercapthoethanol, 50 U/mL penicillin, and 50 μg/mL streptomycin. After 24 hours, activated splenocytes were incubated overnight with retrovirus containing medium in 35 mm diameter wells or T25 flasks (Becton Dickinson Labware, NJ, U.S.A.) coated with recombinant human fibronectin fragment CH-296[18,19] (RetroNectin; Takara Shuzo Co. Ltd., Otsu, Japan). After retroviral exposure, cells were washed and further cultured in IMDM/10% FCS supplemented with 300 IU/mL IL-2. On day 3, cells were either harvested and infused into syngeneic mice, or further cultured until day 8 with addition of fresh IL-2 (300 IU/mL) on days 3 and 6. The murine P815 mastocytoma cell line was grown in IMDM supplemented with 10% FCS, 50 U/mL penicillin, and 50 μg/mL streptomycin. P815 cells and were incubated overnight with retrovirus containing medium in CH-296 coated 35 mm diameter wells. Transduced P815 cells were sorted by flow cytometry to obtain a > 98% ΔLNGFR positive cell line.

Flow cytometric measurements

Cell surface expression of the ΔLNGFR on transduced cells was analyzed by flow cytometry using the murine anti human LNGFR monoclonal antibody (mAb) 20.4 (ATCC) with an indirect fluorescence labeling method. FITC-conjugated goat $F(ab')_2$ anti-mouse IgG and IgM (Tago Immunologics, Camarillo, CA, U.S.A.) was used for staining. CD3, CD4 and CD8 expression was analyzed using R-phycoerythrin (R-PE)-conjugated hamster anti-mouse CD3-ε (PharMingen, San Diego, CA, U.S.A.), FITC-conjugated rat anti-mouse CD4 (L3T4; PharMingen) and FITC-conjugated rat anti-mouse CD8a (Ly-2; Pharmingen) mAb, respectively. Analysis was performed on an Epics XL flow cytometer (Coulter Electronics, Hialeah, FL, U.S.A.).

T cell transfer to syngeneic mice

Fifteen million splenocytes, cultured for 3 or 8 days after ConA/IL-2 stimulation, were injected into the tail vein of C57BL/6 mice in 150 μL Hank's balanced saline solution (HBSS).

DNA isolation

DNA was isolated from whole blood, (transduced) ConA/IL-2 activated splenocytes and (transduced) P815 cells, using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Whole blood samples were pretreated with dextran to remove erythrocytes. Briefly, whole blood was diluted with 3 volumes PBS and incubated with one volume 5% dextran solution (Sigma, St. Louis, MO, U.S.A.). After 30 minutes the upper phase was collected, centrifuged, and the pellet was resuspended in 200 μL PBS. Bone marrow was collected by flushing of the femoral shaft. Organs were resected and representative specimens were taken. DNA was isolated from tissues using the QIAamp DNA Mini Kit (Qiagen).

Real-time quantitative PCR

Cells carrying the provirus were quantified by a real-time PCR analysis[20,21] using the 5' nuclease assay (Taqman)[22] and the ABI/PRISM 7700 sequence detector (Perkin-Elmer Applied Biosystems, Fostercity, CA, U.S.A.)[23,24]. Real-time PCR was performed in a total volume of 50 μL with 1× buffer A, 1.25 U of DNA polymerase (AmpliTaq Gold), 250 μmol/L dNTPs, primers at 300 nmol/L, and dual labeled fluorogenic internal probes at 100 nmol/L. Samples were heated for 10 minutes at 95°C and amplified for 45 cycles of 15 seconds at 95°C and 60 seconds at 60°C. Proviral primers and probe sequences were chosen to specifically amplify a part of the U3 region of any MoMLV-based vector. Provirus primer and probe sequences were as follows: forward; 5'-AAA GAC CCC ACC TGT AGG TTT G-3'; reverse; 5'-TTC CTG ACC TTG ATC TGA ACT TCT CT-3'; probe; 5'-TET (tetrachloro-6-carboxy-fluorescin)-TTA AGT AAC GCC ATT TTG CAA GGC ATG-TAMRA (6-carboxy-tetramethyl-rhodamine)-3'. Mouse serum albumin (Accession: X13060) primer and probe sequences were as follows: forward; 5'-CAA TCC TGA ACC GTG TGT GTC T-3'; reverse; 5'-TTC ATC AAC TGT CAG AGC AGA GAA G-3'; probe; 5'-TET-CCA AGT GCT GTA GTG GAT CCC TGG TGG-TAMRA-3'.

RESULTS

Retroviral transduction of mouse splenocytes with MoMLV-based vector

To analyze T cell survival and migration after infusion into syngeneic recipients, we labeled C57BL/6 splenocytes via retroviral transduction with the MoMLV-based vector SFCM-2 that contains the truncated human LNGFR gene. Splenocytes were activated with ConA and IL-2, retrovirally transduced and subsequently cultured until day 8. To determine transduction efficiency, the percentage of ΔLNGFR positive cells was determined by flow cytometry. We consistently obtained transduction efficiencies between 40-65% (data not shown). These results demonstrate that mouse T cells can be efficiently labeled by retroviral transduction with the MoMLV-based vector SFCM-2 after activation with ConA/IL-2.

Real-time PCR to quantify cells labeled by retroviral transduction

To accurately quantify labeled cells in peripheral blood and tissues, a real-time quantitative PCR detection assay was developed. For this, primers and probe were chosen that specifically amplify a part of the U3 region of the long terminal repeats (LTRs) of the integrated proviral DNA (Fig. 1).

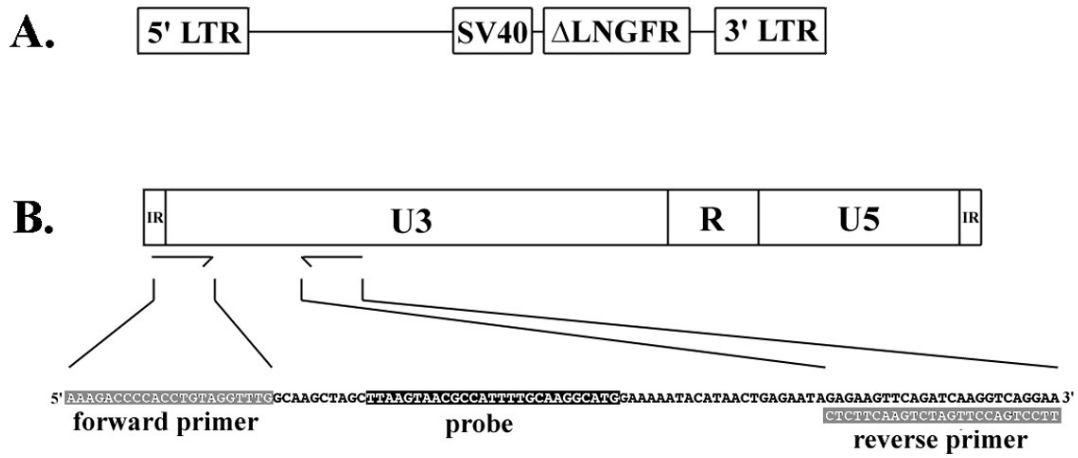

Figure 1. *Position of primers and probe on MoMLV-specific sequence of the proviral DNA. (A) Schematic map of the integrated SFCM-2 proviral genome. ΔLNGFR, human low affinity nerve growth factor receptor cDNA deleted of the intracellular domain; SV40, SV40 early promoter. (B) Position of primers and probe on the U3 sequence of the 5' and 3' long terminal repeat (LTR). The 3' U3 sequence of the SFCM-2 vector acts as a template for both proviral U3 DNA sequences. This results in duplication of the MoMLV-specific target sequence for real-time PCR after integration. IR, inverted repeat; R, direct repeat.*

Amplification of this specific retroviral sequence enables the detection and quantification of cells transduced with any MoMLV-based vector. To determine the amount of input DNA isolated from cell samples collected after infusion, primers and probe were chosen to specifically amplify a part of the mouse albumin gene.

The efficiency of the U3 PCR and mouse albumin PCR was determined by specific amplification of genomic DNA isolated from retrovirally transduced T cells. For this, DNA was serially diluted in water from 750 ng to 7.5 pg. After real-time PCR analysis, initial template concentration was plotted against the cycle threshold (C_t)(Fig. 2).

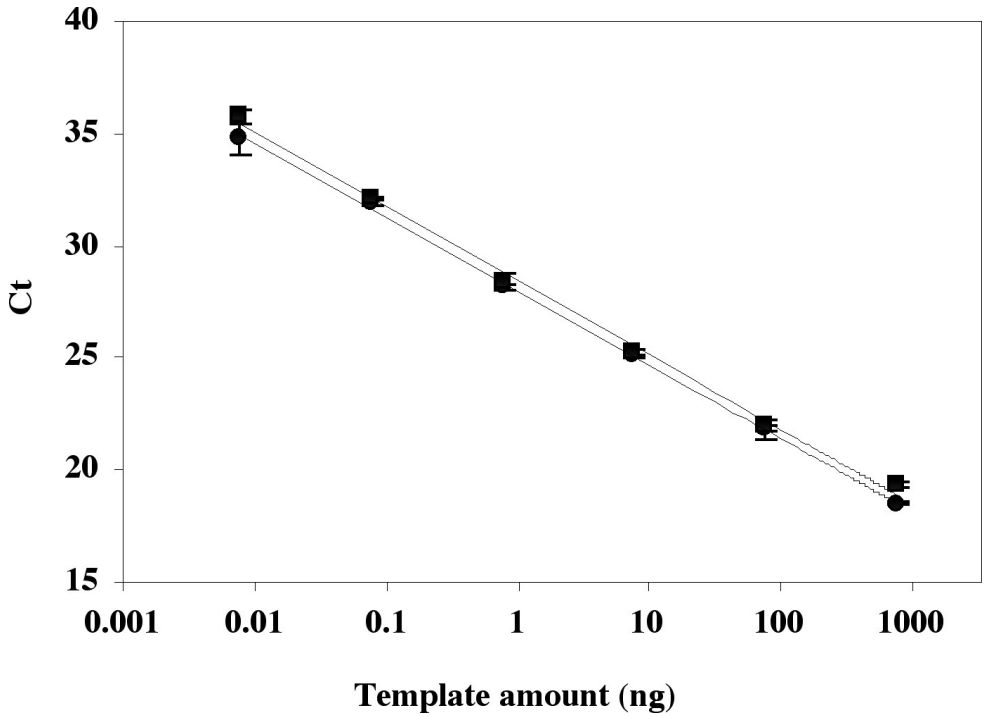

Figure 2. *Amplification efficiencies of mouse albumin PCR and proviral U3 region real-time PCR. The threshold cycles (C_t) after amplification of serial diluted template DNA (i.e. closed squares for U3 region PCR, and closed circles for the mouse albumin gene PCR) are plotted. Regression lines for U3 and mouse albumin are -3.3097 (R^2=0.9968) and -3.2866 (R^2=0.9991), respectively, indicating that both reactions have equal amplification efficiencies.*

The difference in C_t between U3 and albumin DNA amplification (*i.e.* ΔC_t) for each dilution was constant over a dynamic template concentration range of 5 logs. This demonstrates that amplification efficiencies of both real-time PCRs are equal, and, therefore allows normalization of U3 DNA to albumin DNA. The amount of normalized viral DNA in all cell samples was related to a normalized DNA sample obtained from P815 cells expressing ΔLNGFR after retroviral transduction. Using this real-time PCR method, we determined that serially diluted labeled P815 cells in non-transduced cells could be quantified up to at least 0.01% (data not shown). The threshold of detection of labeled cells is up to 0.001% with 750 ng input DNA, as estimated from the data shown in figure 2, assuming that one cell corresponds with 7 pg of genomic DNA. The percentage of labeled P815 cells within serial dilutions of labeled cells in non-transduced cells, as determined by real-time PCR analysis, correlated with the percentage determined by flow cytometry (data not shown).

These results demonstrate that using the developed PCR for specific amplification of the U3 region of MoMLV-based vectors, the percentage of retrovirally labeled cells in blood and tissue samples can be accurately calculated.

Influence of culture period on persistence of infused T cells in peripheral blood

The influence of *in vitro* culture and expansion on the persistence of infused T cells in peripheral blood was investigated by determining the number and retention time of cells cultured 8 days after ConA/IL-2 activation. Therefore, syngeneic C57BL/6 mice (n = 7) were injected with 15×10^6 splenic T lymphoblasts of which 60% were transduced. We found that the amount of labeled cells in blood rapidly decreased to very low frequencies (< 0.2%) within 1 day after infusion (data not shown). In subsequent experiments, we compared the number and retention time of cells cultured 8 days versus cells cultured for only 3 days (Fig. 3). Peripheral blood samples were collected sequentially at 4 hours up to 28 days after adoptive transfer. DNA of white blood cells was isolated and the percentage of labeled cells in these samples was determined by real-time PCR analysis. No significant difference in the amount of labeled cells cultured for 3 days and 8 days was found in peripheral blood 4 hours after infusion (Fig. 3). The amount of labeled cells cultured for 8 days decreased rapidly, as found in the previous experiment. Within one day post infusion the amount of labeled cells was extremely low (< 0.2%) and reached thereafter a steady state level (± 0.05%). In contrast, the number of labeled cells cultured for 3 days increased after infusion and reached a peak level between day 2 and 5 after infusion. Thereafter, the amount of labeled cells decreased to below 0.2% at day 28, but remained significantly higher than the amount of labeled cells cultured for 8 days. These data show that the duration of the culture period of activated T lymphocytes before adoptive transfer has a dramatic effect on the number of T cells that circulate in blood after infusion.

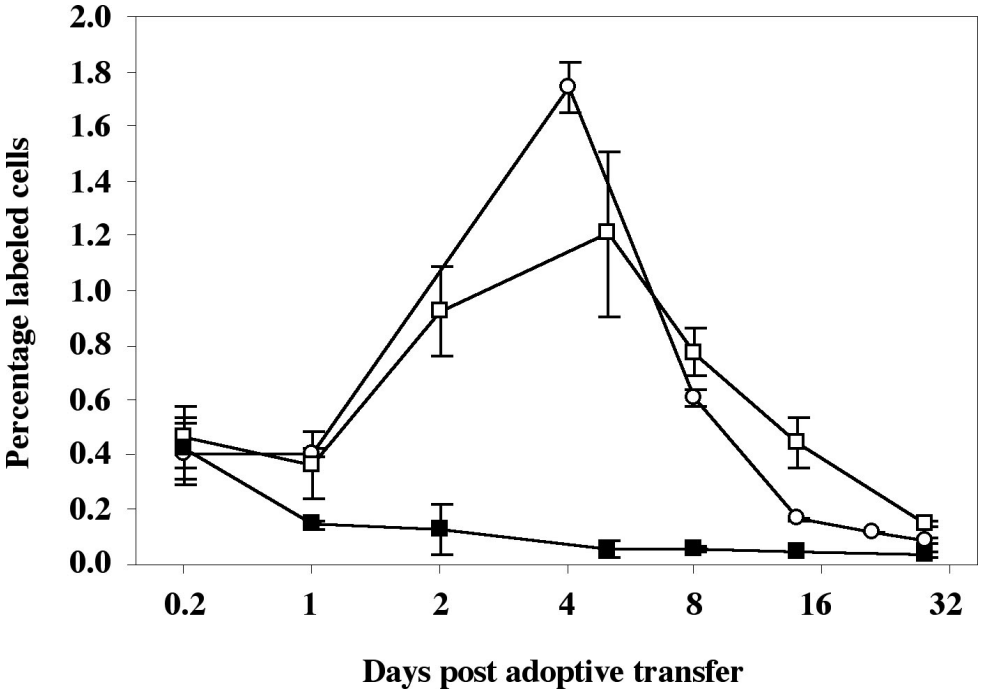

Figure 3. *Percentage of labeled cells in peripheral blood determined by real-time PCR analysis. Before infusion, cells were either cultured for 3 days (open symbols) or 8 days (closed symbols) after ConA/IL-2 stimulation. Mice were infused with 15 × 10^6 splenic T cells of which 50-55% were transduced. Data are means ± SEM. Open and closed squares represent data from 2 mice per time point from; 14 mice were injected with splenocytes cultured for 3 days or 8 days, respectively. Open circles represent data from 4 mice per time point (n = 4) injected with splenocytes cultured for 3 days.*

Influence of culture period of infused T cells on distribution in lymphoid organs

Mature T cells migrate from the bone marrow to lymphoid organs where they become educated and activated. The spleen acts as a reservoir for lymphocytes and the lymph nodes are involved in induction of antigen-specific T cell functions. To investigate the migration to, and persistence of, infused cells in these organs, we determined the percentage of labeled cells in spleen and pooled lymph nodes up to 28 days after infusion (Figs. 4A and B). Four hours after infusion, a higher percentage of labeled cells cultured for 3 days after ConA/IL-2 stimulation could be found in lymphoid organs, compared to labeled cells cultured for 8 days. Thereafter, the percentage of labeled cells cultured for 8 days further decreased to extremely low levels (< 0.1%), whereas the percentage of labeled cells cultured for 3 days increased 4-fold in spleen at day 2, and 70-fold in lymph nodes at day 5. Thereafter, the percentage of labeled cells decreased, but was still detectable in both spleen and lymph nodes 28 days after infusion (0.32 ± 0.02% and 0.84 ± 0.32%, respectively). These results show that a significant percentage of *ex vivo* activated T cells migrate to, and accumulate in, spleen and lymph nodes after infusion in syngeneic mice. Furthermore, these data again demonstrate that the culture period of activated T cells before infusion substantially influences their migration and retention.

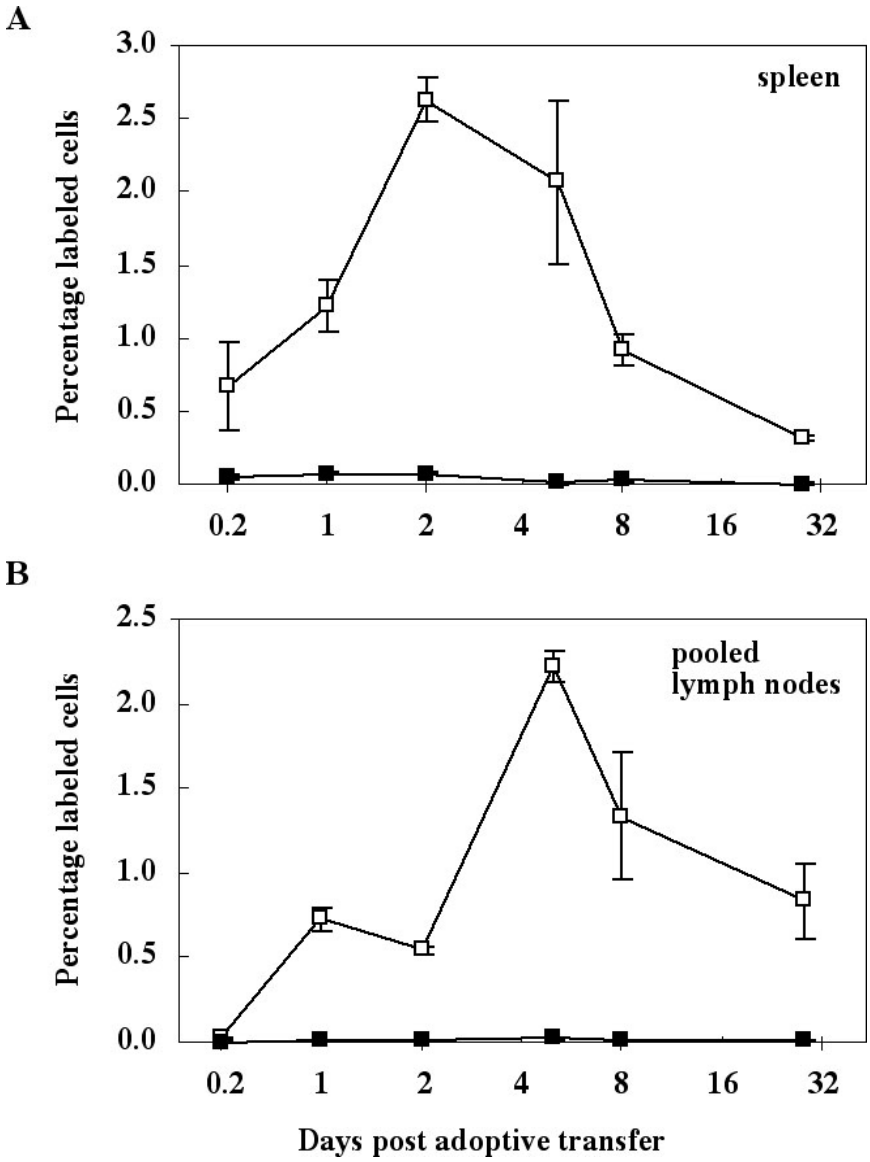

Figure 4. *Percentage of labeled cells in (A) spleen and (B) pooled lymph nodes. Before infusion cells were cultured for 3 days (open squares) or 8 days (closed squares) after ConA/IL-2 stimulation. Data are means ± SEM from 2 mice per time point.*

Influence of culture period of infused T cells on distribution in liver and lungs

In general, activated T cells are preferentially trapped in liver and lungs after infusion, probably due to an increased expression of adhesion molecules in combination with a large amount of capillaries in these organs[25]. Four hours after infusion, a clear quantitative difference of labeled cells was found between cells cultured for 3 and 8 days after ConA/IL-2 stimulation (Figs. 5A and B). This is consistent with our findings in peripheral blood and lymphoid organs. Thereafter, the percentage of labeled cells cultured for 8 days decreased to extremely low levels (< 0.05%). In contrast to the kinetics observed in peripheral blood and secondary lymphoid organs, the percentage of labeled cells cultured for 3 days in both liver and lungs decreased after infusion (Figs. 5A and B).

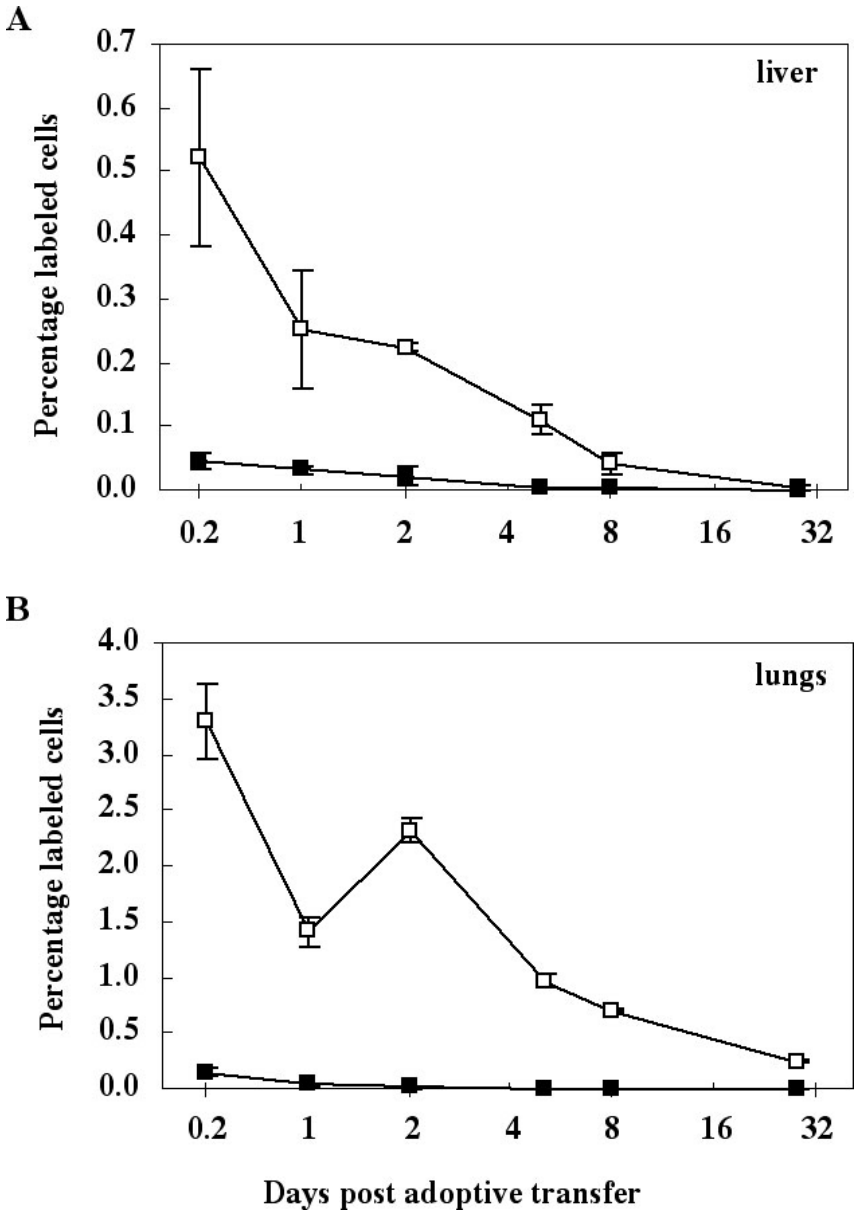

Figure 5. *Percentage of labeled cells in (A) liver and (B) lungs. Before infusion cells were cultured for 3 days (open squares) or 8 days (closed squares) after ConA/IL-2 stimulation. Data are means ± SEM from 2 mice per time point.*

These data show a consistent quantitative effect on the retrieval of infused cells as a result of culture period before infusion. The difference in kinetics of *in vivo* persistence of labeled cells in liver and lungs compared to the kinetics found in peripheral blood and lymphoid organs suggests entrapment of activated T cells rather than migration.

Estimated amount of remaining cells in organs after infusion

To compare the quantitative distribution of labeled cells between different tissues, we determined the amount of labeled cells per tissue. All organs were weighed, except lymph nodes, and the total amount of cells per tissue was estimated by assuming 1 g

of tissue corresponds with 10^9 cells. The total amount of cells in pooled lymph nodes was estimated by the assumption that the total weight of lymph nodes per mouse is 0.08 g[26]. The number of white blood cells per mouse was calculated from the WBC count and the assumption that mice contain 2 mL of blood.

Mice were infused with 15.10^6 splenic T cells of which 50-55% were transduced, so $7.5\text{-}8.25 \times 10^6$ labeled cells were transferred. Four hours after infusion the total amount of retrieved labeled cells that were cultured for 3 days in all tissues analyzed, was approximately 10×10^6. In contrast, only a total number 0.70×10^6 labeled cells in all tissues analyzed could be detected when cells were cultured for 8 days. These estimations of the number of labeled cells present in mice shortly after infusion suggest that cells cultured for 3 days can survive and expand *in vivo*.

Figures 6A and B show the estimated total amount of labeled cells per tissue (*i.e.* blood, spleen, lymph nodes, liver and lung). In mice injected with labeled cells cultured for 3 days, the total number of retrieved cells in all organs was at least a 5- to 10-fold higher than in mice injected with labeled cells cultured for 8 days. Four hours after infusion, the total amount of labeled cells that were cultured for 3 or 8 days in liver and lungs was approximately 8.4×10^6 (84% of total retrieved labeled cells) and 0.57×10^6 (81% of total retrieved labeled cells), respectively. However, the liver to lung ratio after transfer of cells cultured for 8 days was 1.8 and 5 for 4 hours and 1 day, respectively, whereas the liver to lung ratio for cells cultured for 3 days was 0.9 and 1.0 after 4 hours and 1 day after transfer. This indicates that cells cultured for 8 days preferentially accumulate in liver compared to cells cultured for 3 days. From day 1 to day 28 there was a remarkably strong preferential accumulation of labeled cells cultured for 3 days in lymph nodes, whereas there was hardly any accumulation in lymph nodes of labeled cells cultured for 8 days.

These data show a substantial difference in the number of labeled cells that accumulate and migrate to different organs due to culture period before infusion.

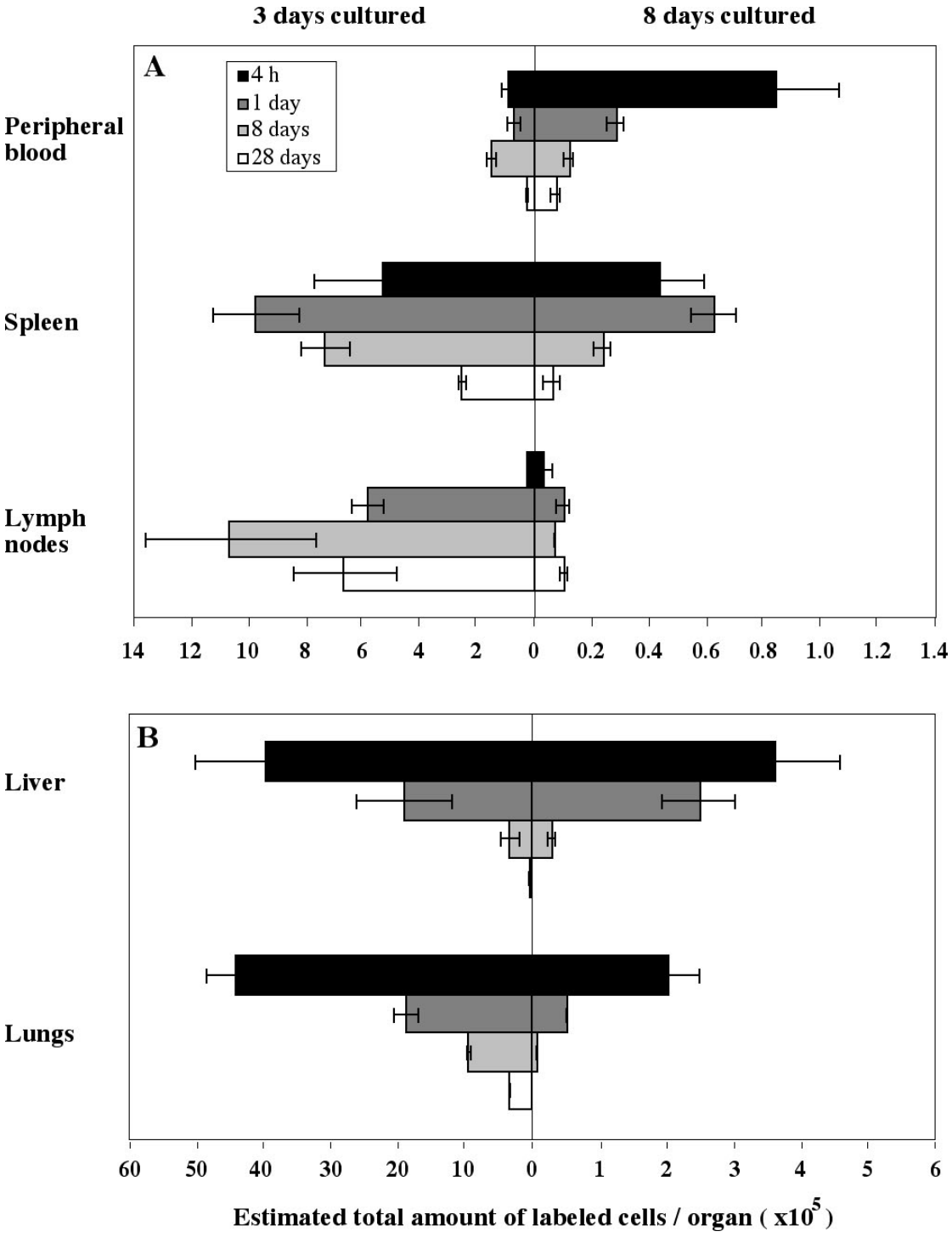

Figure 6. *Estimated amount of labeled cells in (A) peripheral blood, spleen and pooled lymph nodes, and (B) liver and lungs, 4 hours (black bars), 1 day (dark gray bars), 8 days (light gray bars) and 28 days (white bars) after infusion. Left panels show the number of retrieved labeled cells stimulated with ConA/IL-2 for 3 days before infusion, and right panels show the number of retrieved labeled cells stimulated with ConA/IL-2 for 8 days before infusion.*

DISCUSSION

Survival and migration of effector T cells in adoptive immunotherapy are important parameters influencing treatment results. Alterations in phenotype due to activation and culture period before transfer might change these parameters and therefore influence the effector potential of these cells. Tumor-reactive and virus-specific T cells used in adoptive cellular immunotherapy need to be activated and cultured for several days to weeks to induce the required specificity and yield sufficient amounts of effector cells[8-10,27]. Furthermore, genetically engineered T lymphocytes, which have been activated and cultured *ex vivo* to retrovirally transduce them with the HSV-Tk suicide gene, have been used to induce graft-versus-leukemia activity with control of GVHD[28]. In the present report, we studied the persistence and distribution of retrovirally labeled and cultured cells in blood and organs after infusion using a sensitive real-time PCR method.

We have demonstrated a dramatic effect of the period of *in vitro* culture before infusion on the survival time of lymphocytes in syngeneic mice. Cells that were exposed to a 3-day culture period before infusion show a significantly higher persistence in all tissues tested than cells exposed to a 8-day culture period. We are aware that even a short period of *in vitro* activation will influence biodistribution and retention time. However, activation of cells is a prerequisite for retroviral labeling and to ensure a stable integration of provirus in the cells we did not reduce the culture period after retroviral exposure below 3 days, to rule out discrepancies in viral DNA content per cell[29-31].

It has been shown that *ex vivo* activation reduces alloreactive potential of lymphocytes *in vivo*[32]. The reduced reactivity of infused donor lymphocytes is not caused by retroviral transduction *per se* but is a result of cell expansion *in vitro*[32]. Donor lymphocytes transduced with HSV-Tk gene to control the occurrence of GVHD after infusion also show a reduced alloreactive potential in comparison with lymphocytes transferred directly from leukapheresis products. This was demonstrated both in human and animal studies[33,34]. In addition, Contassot *et al.*[35] showed that lymphocytes propagated *in vitro* have to administered at least in a 10-times higher amount to achieve a comparable level of alloreactivity in mice than that observed after infusion of non-cultured lymphocytes. We can extend these observations, showing that the period that lymphocytes have been cultured before infusion strongly influences the persistence and distribution after infusion, which may explain reduced alloreactive potential by cultured cells.

The mechanism by which cells cultured for 8 days are more rapidly cleared from the body remains to be elucidated. It has been shown that clearance of HIV-specific CTL occurs shortly after adoptive transfer[36,37]. This rapid clearance of CTL was suggested to depend on increased vulnerability for apoptosis of these cells after *in vitro* culture[36,37]. We analyzed expression of adhesion molecules on retroviral transduced cells cultured for 3 and 8 days. We only found a slight increase of LFA-1 and CD2 expression on T cells cultured for 8 days, suggesting that the expression level of adhesion molecules does not dominantly contribute to the observed differences in *in vivo* persistence and distribution (data not shown). In addition, we studied cell proliferation by measurement of DNA contents of cells after a 3- and 8-day culture period. Cells cultured for 8 days showed fewer proliferating cells (± 52% and ± 37%

of cells in S/G_2M-phase, respectively). This decreased cell cycling activity might influence long term persistence of infused cells but might have limited effect on cell numbers retrieved from the mice shortly after infusion.

The liver seems the site at which apototic cells accumulate[38,39], whereas retention in lungs represents an intrinsic component of normal lymphocyte circulation[40]. We analyzed the relative distribution and retention of labeled cells in several organs after infusion and showed that the highest number of labeled cells was found in liver and lung. Moreover, we found that cells cultured for 8 days after ConA/IL-2 stimulation preferentially accumulate in liver compared to cells cultured for 3 days. This difference was already significant 4 hours after infusion, further increased 1 day after of infusion, and remained significant up to 28 days after infusion. These data suggest that cells cultured for 8 days are more vulnerable for apoptosis and, therefore, are trapped in the liver shortly after infusion for clearance. The relatively high percentage of labeled cells cultured for 3 days in lungs, shortly after infusion, may indicate that a higher percentage of these cells still circulate in the body.

The most dramatic difference on the *in vivo* retention of infused cells caused by the culture period was found in the lymph nodes. We found a 70-fold higher amount of cells cultured for 3 days than cells cultured for 8 days. The highest amount of labeled cells in lymph nodes was found at day 5. Interestingly, we observed a similar accumulation pattern of labeled cells in lymph nodes and peripheral blood. This confirms the observation of Vasseur *et al*.[41], who found that quantitative changes of *in vivo* activated T cells in blood reflect data obtained from lymph nodes, and, therefore, suggest that peripheral blood samples are useful to monitor immune responses in human studies.

Several groups have stressed the importance of using the means to quantify survival of transferred effector cells *in vivo*[27,36,42,43]. For the detection of retrovirally marked cells, we designed primers and probes for MoMLV-specific DNA sequences. This enables detection of cells marked with any MoMLV-derived retroviral vector, in contrast to the use of markergene-specific sequences to detect and quantify labeled cells[44-46]. Retroviral labeling enables monitoring of infused cells *in vivo* for a long period due to stable integration of the proviral DNA. All daughter cells of transduced cells will contain proviral DNA, as a result, even after proliferation and division cells can be detected. These characteristics of retroviral labeling provide a strong advantage over the use of radioisotopes or fluorescent dyes in labeling procedures of cells. Isotope half-life, leakage of dye out of cells and reduction of label in dividing cells will influence quantification and significantly decrease the follow-up time after transfer.

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CHAPTER 3

Monitoring of developing graft-versus-host disease mediated by Herpes Simplex Virus thymidine kinase gene-transduced T cells

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ABSTRACT

Introduction of the HSV-Tk suicide gene in allogeneic T cells offers the possibility to control developing host-reactive cells within the context of allogeneic bone marrow transplantation (BMT). Sensitive quantitative detection methods are a prerequisite to follow genetically modified T cells both in peripheral blood as well as in tissues to study their involvement in graft-versus-host disease (GVHD)-induced lesions as well as their disappearance or persistence after ganciclovir (GCV)-induced suicide. We monitored the alloreactivity of HSV-Tk-transduced T cells after BMT by studying their *in vivo* distribution and quantity in peripheral blood and in tissues in a WAG/Rij into Brown Norway fully mismatched rat allogeneic BMT model. Genetically modified T cells were quantified in blood and tissues by fluorescence-activated cell sorting, immunohistochemical, and real-time quantitative polymerase chain reaction (PCR) analysis. A significant increase in the number of allogeneic HSV-Tk$^+$ T cells was found in particular in spleen and lymph nodes and large numbers were found in tongue, skin and intestines. In blood, an increase of HSV-Tk$^+$ T cells closely preceded clinical symptoms of GVHD. Real-time quantitative PCR proved to be a fast and accurate tool by which to quantify transduced T cells both in blood and tissues. This enables the study of the *in vivo* alloreactivity of retrovirus-transduced cells and the response of HSV-Tk-expressing T cells to GCV-induced suicide therapy. Furthermore, we showed the potential use to study specific cause-effect relationships in a broad range of animal and clinical studies involving genetically engineered cells.

OVERVIEW SUMMARY

We monitored the alloreactivity of retrovirally transduced T cells after BMT in an allogeneic rat transplantation model. Genetically modified T cells were quantified in blood and tissues using fluorescence-activated cell sorting, immunohistochemical analysis, and real-time quantitative PCR analyses. We found an increased infiltration of HSV-Tk T cells in GHVD target organs in allogeneic transplanted rats compared with syngeneic transplanted rats. In blood, an increase of allogeneic HSV-Tk$^+$ cells closely preceded clinical symptoms of GVHD. Immunohistochemical analysis revealed active involvement of HSV-Tk$^+$ cells in developing GVHD lesions in a variety of tissues. Comparison of detection methods showed that real-time PCR analysis enabled fast and accurate quantification of transduced cells both in blood and tissues.

INTRODUCTION

ALLOGENEIC BONE MARROW TRANSPLANTATION (BMT) is a frequently used treatment modality to cure patients with leukemia. Despite matching for major histocompatibility antigens there is a substantial risk for serious morbidity and mortality caused by graft-versus-host disease (GVHD)[1]. T cells present in the donor stem cell graft are responsible for this complication[2-4]. A number of techniques exist for removing T cells before BMT, most of which use antibodies (complement-mediated lysis, immunotoxins, and immunomagnetic beads) or physical methods (soybean lectin agglutination, counterflow elutriation and albumin gradient fractionation). Clinical studies using these approaches have unambiguously shown that T cell depletion markedly reduces the incidence and severity of GVHD. However, T cell depletion is associated with an increased rate of severe and often fatal infections, a higher incidence of graft rejection, and an increased risk of leukemia recurrence. Thus, alloreactive T cells in the graft can have not only a negative effect, that is, induction of GVHD, but also a positive effect, that is, graft-versus-leukemia (GVL) activity. An alternative approach to maximize the beneficial effects of GVL activity, while minimizing the risk of severe GVHD, is the genetic modification of donor T cells by introduction of the herpes simplex virus thymidine kinase (HSV-Tk) suicide gene through retroviral transduction[5]. T cells expressing HSV-Tk are approximately 10,000-fold more sensitive to the prodrug ganciclovir (GCV) than are normal cells[6]. *Ex vivo* engineered T cells expressing HSV-Tk thus can selectively be eliminated after administration of ganciclovir in case life-threatening GVHD develops. A number of phase I/II trials with HSV-Tk suicide gene-transduced T cells in order to modulate GVHD have been carried out[7-10]. In these trials all patients that developed GVHD showed improvement of clinical signs after GCV administration. Still, basic questions regarding the involvement of genetically modified T cells in the development of GVHD-induced lesions, as well as their disappearance or persistence after GCV-induced suicide, remain unsolved.

We address these questions using an MHC-mismatched allogeneic rat transplantation model originally described by Kloosterman *et al.*[11,12], in which the WAG/Rij rat serves as allogeneic BM donor for the Brown Norway (BN) rat. Lethally irradiated BN rats were given suicide gene-transduced T cells of WAG/Rij origin simultaneously with BMT. In these allogeneic recipients severe GVHD developed. As a syngeneic control WAG/Rij rats received the same treatment. Genetically modified T cells were quantified in blood and tissues of allogeneic and syngeneic recipients. In blood, quantification was performed by fluorescence-activated cell sorting (FACS) and real-time polymerase chain reaction (PCR). In tissues, quantification was performed by real-time PCR and after immunohistochemical staining.

We show the involvement of retrovirally transduced T cells in the development of GVHD. This was demonstrated by a rapid increase in the number of allogeneic HSV-Tk-transduced T cells that closely preceded clinical signs of GVHD. Furthermore, we found increased numbers of allogeneic HSV-Tk-labeled T cells, compared with syngeneic HSV-Tk$^+$ T cells, in all tissues. In GVHD target organs, almost all T cells were HSV-Tk positive. Real-time PCR proved to be the fastest and most sensitive tool for the quantification of retrovirally transduced T cells in both peripheral blood and tissues. Thus, this method provided an accurate tool to study the involvement of transduced T cells during induction and perpetuation/progression of an alloresponse.

Furthermore, its high sensitivity will enable us to monitor the disappearance or persistence of HSV-Tk-transduced T cells after GCV-induced suicide.

MATERIALS AND METHODS

Animals

Experiments were carried out with Brown Norway (BN)/RijHsd (*RT-1A*^{n}) and WAG/RijHsd (*RT-1A*^{u}) rats. Animals were kept in filter top cages and received sterilized food and acidified water *ad libitum*.

Bone marrow transplantation

Bone marrow cells were collected by flushing of the cavity of WAG/Rij rat tibiae and femora with RPMI (GIBCO-BRL, Bethesda, MD). A single-cell suspension was obtained by filtering the bone marrow through a cell strainer (Falcon; BD Biosciences Discovery Labware, Bedford, MA). BMT recipients received an equivalent dose of 7.2 Gy of γ rays in total body irradiation (TBI), using a linear accelerator approximately 6 hr before BMT. TBI-conditioned BN and WAG/Rij rats received a BM transplant of 5×10^7 bone marrow cells. The BM was not T cell depleted and contained 3% R73-positive cells, which amounted to 1.5×10^5 T cells. The BM transplant was supplemented with 2×10^7 genetically modified T cells. The BM and the genetically modified T cells were both of WAG origin.

Animal handling

Body weights of the animals were recorded at regular intervals as a relevant parameter by which to monitor the onset and further course of GVHD[12]. At various time points after the BMT blood samples were taken from the tail vein for flow cytometric staining and isolation of genomic DNA. Animals were killed at day 18, before the allogeneic transplanted animals became moribund, and a variety of tissues were collected for isolation of genomic DNA and for immunohistochemical staining.

Cell culture

WAG/Rij rat spleen cell suspensions were made by gently pressing spleens through sterile filter chambers (NPBI, Emmer-Compascuum, The Netherlands). Lymphocytes were isolated from the single-cell suspension by centrifugation on a Ficoll density gradient (d = 1.077 g/cm^3) (Amersham Pharmacia, Uppsala, Sweden). Subsequently, T cells were activated with concanavaline A (ConA, 5 μg/ml; Boehringer Mannheim, Mannheim, Germany) in Iscove's modified Dulbecco's medium (IMDM; GIBCO-BRL) supplemented with human recombinant interleukin-2 (rIL-2, 300 IU/ml; Chiron, Amsterdam, The Netherlands), 10 % fetal calf serum (FCS; Integro, Zaandam, The Netherlands), penicillin (100 U/ml), streptomycin (100 μg/ml), 2 m*M* L-glutamine (GIBCO-BRL) and 5×10^{-5} *M* 2-mercaptoethanol (Merck, Darmstadt, Germany) and incubated at 37ºC in a 5% CO_2 atmosphere. After 24 hr ConA was washed away and activated splenocytes were cultured in IMDM medium supplemented as mentioned above.

The ecotropic packaging cell line Phoenix[13] was cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL) supplemented with 10 % FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 2 m*M* L-glutamine.

Retroviral gene transduction

LZRS-TN is a for Moloney Murine Leukemia Virus (MoMLV)-based ecotropic retroviral vector comprising the HSV-1 Tk gene, the simian virus 40 (SV40) promoter, and the truncated human nerve growth factor receptor (NGFR) in the LZRS vector[13]. The HSV-1 Tk gene, the SV40 promoter and truncated NGFR were isolated from SFCMM-3 retroviral vector [5]

Retroviral particles were generated after $CaPO_4$ transfection of the ecotropic packaging cell line Phoenix with 20 μg of LZRS-TN (GIBCO-BRL). Twenty-four hours after transfection, medium was replaced by 10 ml of fresh medium. The following day, the retroviral supernatant was harvested, filtered through a 0.45-μm pore size filter (Millipore, Bedford, MA), and frozen. Fresh medium was added to the cells for repeated harvesting of viral supernatant. After two or three rounds of supernatant harvesting, Phoenix cells were cultured for 3 days in the presence of puromycin (1 μg/ml; Sigma, St. Louis, MO) followed by 2 days in medium without puromycin before final harvesting of retroviral supernatant.

Before transduction, non-tissue culture-treated flasks (Falcon) were coated with RetroNectin (12,5 μg/ml; Takara Shuzo, Otsu, Shiga, Japan) for 2 hr, blocked with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 30 min, and washed three times with PBS. Retroviral supernatant containing the LZRS-TN retroviral vector was added to RetroNectin-coated flasks and preincubated for 1 hr. Subsequently, ConA-activated WAG/Rij T cells were added at a concentration of 2×10^6 cells/ml and incubated overnight. T cells were then harvested and transferred into fresh medium. Fresh retroviral supernatant was added to flasks and preincubated for 1 hr. Harvested WAG/Rij T cells were readded to the flasks with retroviral supernatant. After 6-12 hr this was repeated. After retroviral exposure cells were washed and cultured in supplemented IMDM.

Flow cytometry

T cell receptor and NGFR membrane expression on WAG/Rij T cells was analysed with the monoclonal antibodies R73 (Instruchemie, Hilversum, The Netherlands) and 20.4 (culture supernatant, a gift of MolMed, Milan, Italy), respectively. Cells were washed in PBS containing 1% FCS. Subsequently, 20 μl of diluted monoclonal antibody (mAb) was added to the cell pellet and cells were incubated for 30 min at 4°C. Cells were washed in PBS-1% FCS. Phycoerythrin (PE)- or fluorescin isothiocyanate (FITC)-conjugated goat anti-mouse (GAM) IgG was added for detection by indirect fluorescence and cells were incubated for 30 min at 4 °C. After incubation cells were washed in PBS-1% FCS and resuspended in 0.5 ml of PBS for analysis on a FACSCalibur flow cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA).

Immunomagnetic purification of gene-transduced WAG/Rij T cells

The truncated NGFR gene carried by LZRS-TN encodes a nonfunctional form of the human low-affinity receptor for nerve growth factor. NGFR is located on the cell surface and allows selection of gene-transduced cells. Retrovirally transduced cells were harvested on day 5, washed in PBS-1% FCS-1 m*M* EDTA, and labeled in with anti-NGFR mAb 20.4 (20 μl/10^6 cells) for 15 minutes on ice.

Cells were washed in PBS-1% FCS-1 m*M* EDTA and GAM IgG microbeads (20 μl/10^7 cells, Miltenyi Biotec, Bergisch Gladbach, Germany) were added for 10 min at 4°C. Cells were washed in PBS -1% FCS-1 m*M* EDTA and NGFR-positive cells were separated on a magnetic activated cell-sorting (MACS) column according to the manufacturer protocol (Miltenyi Biotec). The purity of the selected population was determined by flow cytometry after incubation with PE-conjugated GAM Ig. The total duration of the transduction and selection procedure was 5 days and resulted on average in 45% of the T cells expressing NGFR. The purity of the MACS-selected population, measured by FACS, was 95%, implying that from the infused number of 2×10^7 T cells, 1×10^6 cells are HSV-Tk/NGFR negative. HSV-Tk^+ T cells that are produced according to this protocol retain their full potential to induce a lethal GVHD in the allogeneic transplantation setting that was used[6].

Immunohistochemistry

Rats were killed on day 18 after BMT, using Euthesate (CEVA Santé Animale, Maassluis, The Netherlands). Small blocks of tissues were collected and frozen in liquid nitrogen. Tissues were bedded into Tissue-tek (Sakura Finetek, Torrance, CA) and slices of 6 μm were cut on a cryostat, air-dried, and fixed in acetone. Staining was performed with a DAKO animal research kit (ARK/horseradish peroxidase [HRP]) (Dako Corporation, Carpinteria, CA, USA) with extra avidin and biotin blocking steps, each for 15 minutes (avidin/biotin blocking kit; Vector Laboratories, Burlingame, CA). The following antibodies were used: anti-NGFR, (20.4; 1.5 μg/ml) and anti-T cell receptor (R73; ascites, diluted 1:60,000). Immunostaining was followed by hematoxylin staining. Positive cells are recognized on the basis of a brown discoloration. The percentage of positive cells was determined by estimating the frequency of brown cells in five or six representative microscopic fields.

DNA isolation

DNA was isolated from whole blood and (transduced) ConA/IL-2 activated splenocytes, using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). Whole blood was collected from the tail vein into sodium citrate (3.8%; 10:1[v/v]) and samples were pretreated with dextran to remove erythrocytes. Briefly, whole blood was diluted with 3 volumes of PBS and incubated with 1 volume of 5% dextran solution (Sigma) at room temperature. After 30 minutes the upper phase was collected and centrifuged, and the pellet was resuspended in 200 μl of PBS.

Organs were dissected from the animals and representative specimens were taken. Bone marrow was collected by flushing the femoral shaft with medium. DNA was isolated from tissue and bone marrow, using the QIAamp DNA Mini Kit (Qiagen).

Real-time quantitative PCR

The number of HSV-Tk/NGFR-transduced lymphocytes in the peripheral blood and in various organs were quantified by a real-time PCR analysis[14,15] using the 5' nuclease assay (TaqMan; Applied Biosystems, Fostercity, CA)[16] and the ABI/PRISM 7700 sequence detector (Applied Biosystems)[17].

Real-time PCR was performed in a total volume of 50 μl with 1 × buffer A, 1.25 U of DNA polymerase (AmpliTaq Gold; Applied Biosytems), 250 μM dNTPs, primers at 300 nM and dual-labeled fluorogenic internal probes at 100 nM. Samples were heated for 10 min at 95°C and amplified for 45 cycles of 15 seconds at 95°C and 60 seconds at 60°C. The proviral primers and probe sequences chosen specifically amplify a part of the U3 region of the long terminal repeat (LTR) of the integrated proviral DNA. Provirus primer and probe sequences were as follows: forward, 5'-AAA GAC CCC ACC TGT AGG TTT G-3'; reverse, 5'-TTC CTG ACC TTG ATC TGA ACT TCT CT-3'; probe, 5'-TET (tetrachloro-6-carboxy-fluorescin)-TTA AGT AAC GCC ATT TTG CAA GGC ATG-TAMRA (6-carboxy-tetramethyl-rhodamine)-3'. Rat preproalbumin (derived from the messenger RNA sequence for rat preproalbumin[18], GenBank accession number V01222) primer and probe sequences were as follows: forward, 5'-AGT GAG CGA GAA GGT CAC CAA-3'; reverse, 5'-CGT CAA CTG TCA GAG CAG AGA AA; probe, 5'-TET-CCG TCT TTC CAC CAA GGA CCC ACT ACA-TAMRA-3'. Amplification efficiencies were determined by specific amplification of the proviral amplicon as well as the rat preproalbumin locus, using a serial dilution of transduced T cell DNA in distilled H_2O, ranging from 500 ng to 5 pg of genomic DNA. Initial template concentrations were related to cycle threshold (C_t). The difference in C_t values (ΔC_t) for each dilution was constant over a dynamic template concentration range of 5 logs, demonstrating that the amplification efficiencies in both reactions were equal. Because the C_t is proportional to the initial template concentration, the ΔC_t relates the amount of transduced cells (C_t of proviral DNA) to the total amount of cells used in the amplification reaction (C_t of rat preproalbumin). This normalized ΔC_t value was used for accurate relative quantification of Tk-transduced cells using the comparative C_t method (*ABI PRISM 7700 Sequence Detection System: User Bulletin #2*; Applied Biosystems). The percentage of Tk-transduced cells was determined by reference to a calibrator DNA sample of purified HSV-Tk/NGFR-transduced T cells (purity ≥ 95%). For peripheral blood absolute numbers of HSV-Tk/NGFR-positive T cells per milliliter were calculated by correction for white blood cell (WBC) counts. The sensitivity of the PCR assay was between 10^{-4} and 10^{-5}.

RESULTS

Induction of GVH reactivity

To study the role of HSV-Tk-transduced T cells in GVHD after allogeneic BMT, we induced alloreactivity in BN rats by injection of HSV-TK/NGFR-transduced WAG/Rij T cells simultaneously with WAG/Rij BM after lethal irradiation. Syngeneic WAG/Rij rats received the same treatment to study dissemination of HSV-Tk^+ T cells in peripheral blood and tissues in the absence of allostimulation. Body weights of the transplanted rats were measured at regular time intervals after BMT as a parameter to determine the onset and progression of GVH reactivity (Fig. 1).

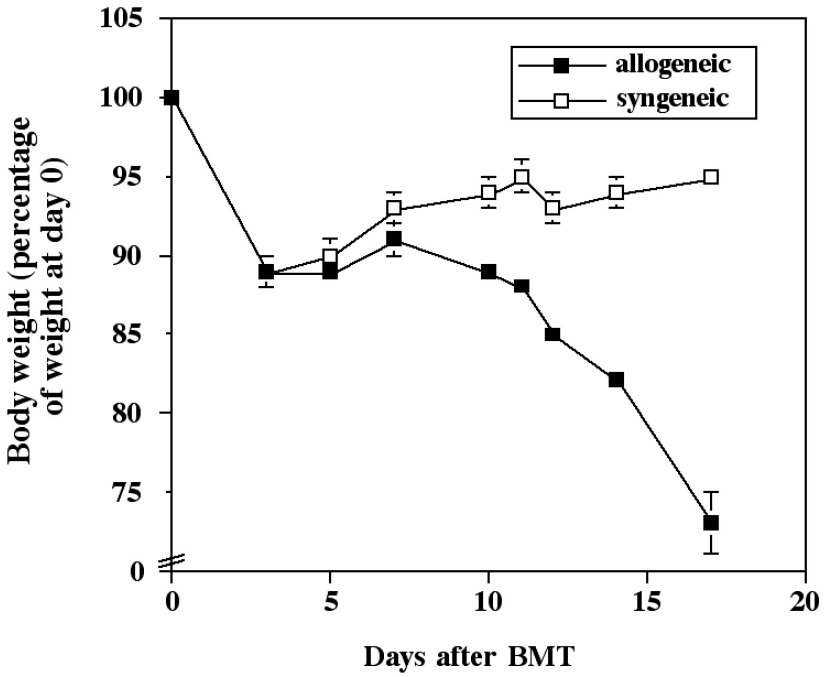


Figure 1. *Relative body weights of allogeneic and syngeneic transplanted rats after BMT. Body weights of allogeneic (filled squares) versus syngeneic (open squares) transplanted rats were measured and plotted as percentage of body weight at day 0. Data repesents mean of four animals per group ± SE.*

Allogeneic as well as syngeneic recipients showed an initial drop in body weight due to the BMT procedure. The body weights of the allogeneic recipients decreased from day 7 onward, which continued until day 18, when animals were killed for analysis of tissue architecture and T cell infiltration. In contrast, syngeneic recipients regained normal weight and recovered without complications. From day 12 onward, allogeneic recipients developed clinical symptoms of GVHD, such as rash on paws and snout, hunched posture, ruffled fur, hair loss, and diarrhea, which gradually increased in severity. Furthermore, resection of tissues showed macroscopic reduction in liver and spleen size. The alloreactive responses that occur in this model enable us to monitor the amount of HSV-Tk-transduced T cells and their *in vivo* distribution during GVHD development.

Quantification of HSV-Tk/NGFR-transduced T cells in the peripheral blood

We determined the percentage and absolute numbers of HSV-Tk/NGFR-transduced T cells in peripheral blood of rats after allogeneic and syngeneic BMT. Blood samples were taken at regular time intervals for flow cytometric and real-time PCR analysis. FACS analysis showed that the majority of T cells in the circulation were HSV-Tk positive, that is, on days 7, 10, and 14 these values were, 63, 69, and 44% for the allogeneic transplanted group versus 70, 79, and 56% for the syngeneic group. Analysis of the absolute number of HSV-Tk/NGFR T cells in the peripheral blood of allogeneic and syngeneic recipients by either FACS or real-time PCR revealed that there was no difference through day 10 (Fig. 2A and B).

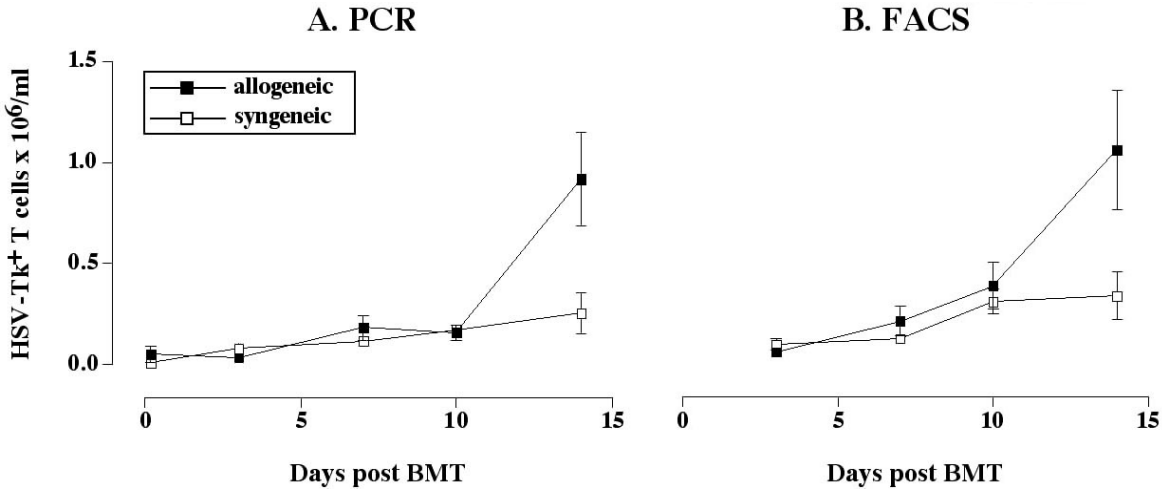


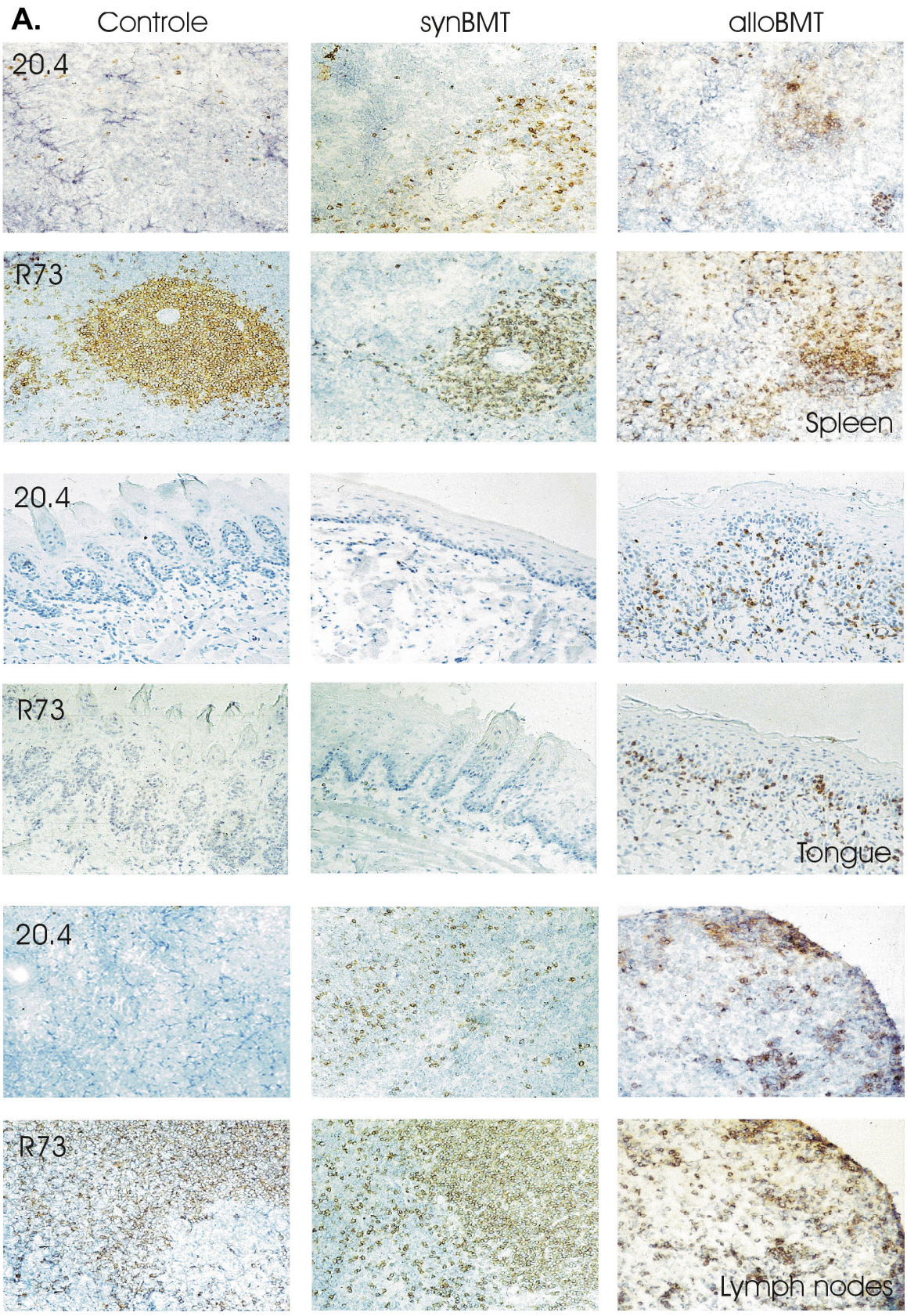
Figure 2. *Absolute number of HSV-Tk/NGFR T cells (× 10^6/ml) in peripheral blood of allogeneic and syngeneic transplanted rats after BMT, measured by real-time PCR (A) and flow cytometry (B). Data represents average values of four animals per group ± SEM.*

Thereafter, the number of HSV-Tk/NGFR T cells in rats that had received an allogeneic BMT increased, whereas the number of HSV- Tk^+ T cells in syngeneic recipients remained at a continuous level. The increase of HSV-Tk^+ T cells from day 10 after allogeneic BMT correlates with the decrease in body weight, which is considered to be an early sign of GVHD (Fig. 1). In addition, Fig. 2 shows that quantification of HSV-Tk/NGFR T cells in peripheral blood by real-time PCR agreed well with flow cytometric analysis. However, shortly after BMT flow cytometric analysis could not be performed because of the low WBC numbers due to a transplantation-related aplastic phase (Fig. 2B). In conclusion, these data show that GVH reactivity in allogeneic recipients is, at least in part, exerted by HSV-Tk-transduced T cells, and that this alloreactivity is reflected by an increase in these cells in the peripheral blood.

Involvement of HSV-Tk/NGFR-transduced T cells in GVH-induced lesions: Infiltration of T cells and HSV-Tk/NGFR$^+$ T cells in tissues

To examine the involvement of HSV-Tk/NGFR-transduced T cells in GVH-induced lesions, we examined the localization of T cells and HSV-Tk/NGFR-transduced T

cells in frozen tissue sections of a representative allogeneic transplanted rat, 18 days after BMT, by immunohistochemical staining. T cell localization in the allogeneic transplanted rat was compared with that in a syngeneic transplanted WAG/Rij and untransplanted BN rat. From each rat, eight different tissues were investigated, that is, spleen, lymph node, tongue, skin, intestine, liver, lung, and kidney. In allogeneic transplanted rats, normal tissue architecture was severely damaged, whereas tissue architecture in syngeneic transplanted rats was intact and showed post-BMT histological recovery of lymphoid tissues. An example of the latter is found in the spleen of the syngeneic transplanted rat in which a follicle structure is recognizable which contains transduced as well as nontransduced T cells (Fig. 3). In the allogeneic transplanted rat there was a clear infiltration of both transduced and nontransduced allogeneic T cells in the various tissues, particularly in tongue, skin, intestine, lymph node, and spleen. In tongue and skin, we observed a predominant T cell localization in the subepithelial regions. Notably, in GVHD target tissues the majority of the T cells were HSV-Tk/NGFR positive. In lung, and kidney T cells were present at lower frequencies, and HSV-Tk/NGFR-positive cells were rarely found (data not shown). These results show that tissue damage in allogeneic transplanted rats coincides with a clear T cell infiltration, including HSV-Tk/NGFR-transduced cells, illustrating the involvement of transduced T cells at the site of GVHD-induced lesions.


A.
Controle
synBMT
alloBMT
20.4
R73
Spleen
20.4
R73
Tongue
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R73
Lymph nodes

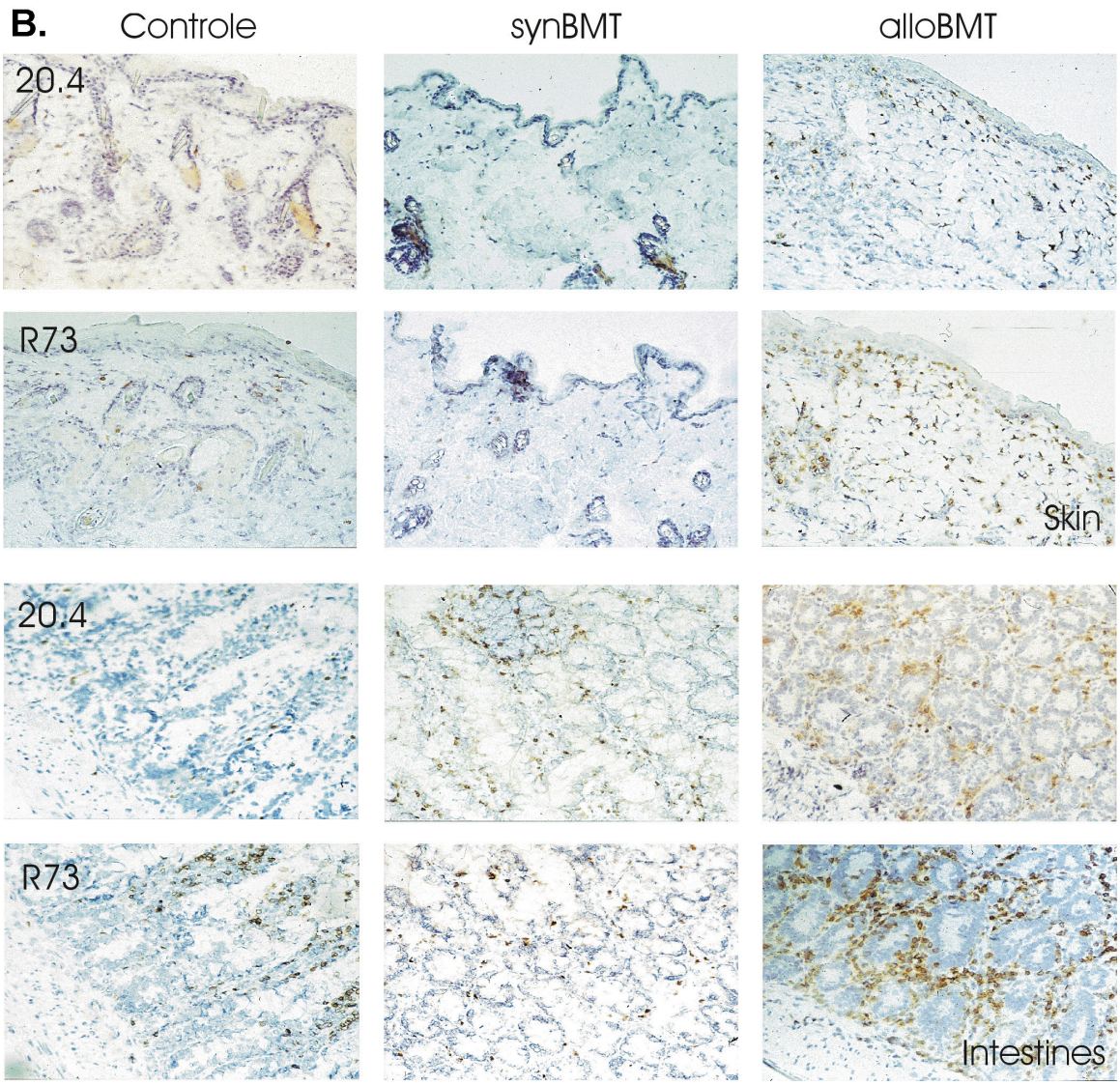

Figure 3. *Immunohistochemical analysis of the infiltration of T cells and HSV-Tk/NGFR-transduced T cells in spleen, lymph node, and tongue (**A**), and skin and intestine (**B**), of allogeneic and syngeneic transplanted rat 18 days after BMT. T cells were stained with R73 mAb, and NGFR-expressing cells were stained with 20.4 mAb. Positive cells show a brown color.*

Involvement of HSV-Tk/NGFR-transduced T cells in GVH-induced lesions: Quantification of HSV-Tk/NGFR$^+$ T cells in the tissues

To estimate the amount of T cell infiltration in GVH-induced lesions, we determined the percentage of R73-positive cells (total T cells) as well as the number of 20.4-positive cells (HSV-Tk-transduced T cells) after immunohistochemical staining. However, exact quantification was complicated by the fact that GVH-induced cell damage leads to aspecific background staining that is particularly disturbing at low levels of T cell infiltration (*i.e.*, less than 1-2%). To accurately quantify the amount of HSV-Tk/NGFR T cells in the various organs, we performed real-time PCR analysis on genomic DNA of representative samples. Beside the aforementioned tissues, brain and bone marrow were also included. Figure 4 shows that in all organs examined the percentage of HSV-Tk/NGFR-transduced T cells was higher in the allogeneic

transplanted animals compared with syngeneic transplanted rats. High percentages of HSV-Tk/NGFR T cells were found in tongue, skin, intestine, spleen and lymph node. Percentages below 5% were found in kidney, liver, brain and BM. The high ratio of allogeneic over syngeneic HSV-Tk/NGFR T cells in liver and bone marrow was suggestive of alloreactivity of these cells in these tissues.

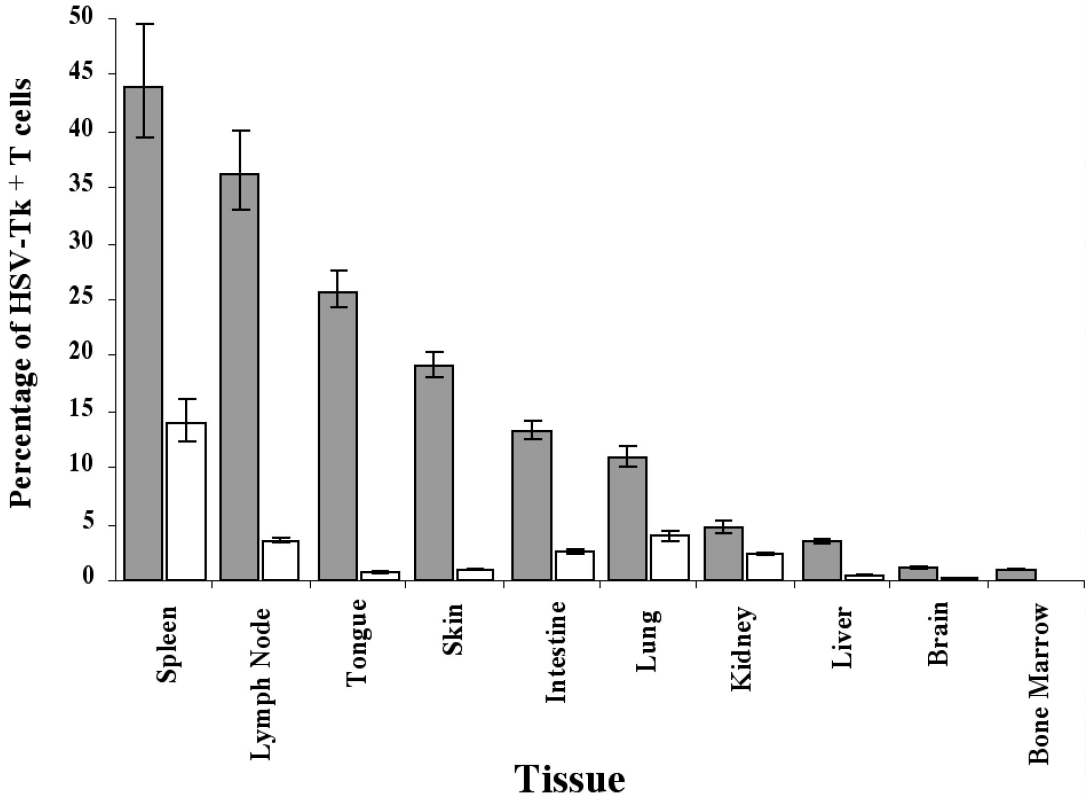

Figure 4. *Percentage of HSV-Tk/NGFR T cells in various tissues of allogeneic (gray columns) and syngeneic (open columns) transplanted rats 18 days after BMT, measured by real-time PCR. Data represent average values of four animals per group ± SEM.*

Figure 5 shows a comparison of data obtained through immunohistochemical and real-time PCR analysis for the same allogeneic and syngeneic transplanted representative rats. BN and WAG/Rij untransplanted rats were included as controls. In the syngeneic transplanted rat there was no discrepancy in the low levels of HSV-Tk^+ T cells found by immunohistochemical and real-time PCR analysis. In the allogeneic transplanted rat a higher percentage of HSV-Tk positive T cells was found by real-time PCR analysis in spleen, tongue, kidney and lung. The percentage of HSV-Tk-positive T cells determined by real-time PCR did not exceed the percentage of total T cells as determined by immunohistochemical staining, except in lung.

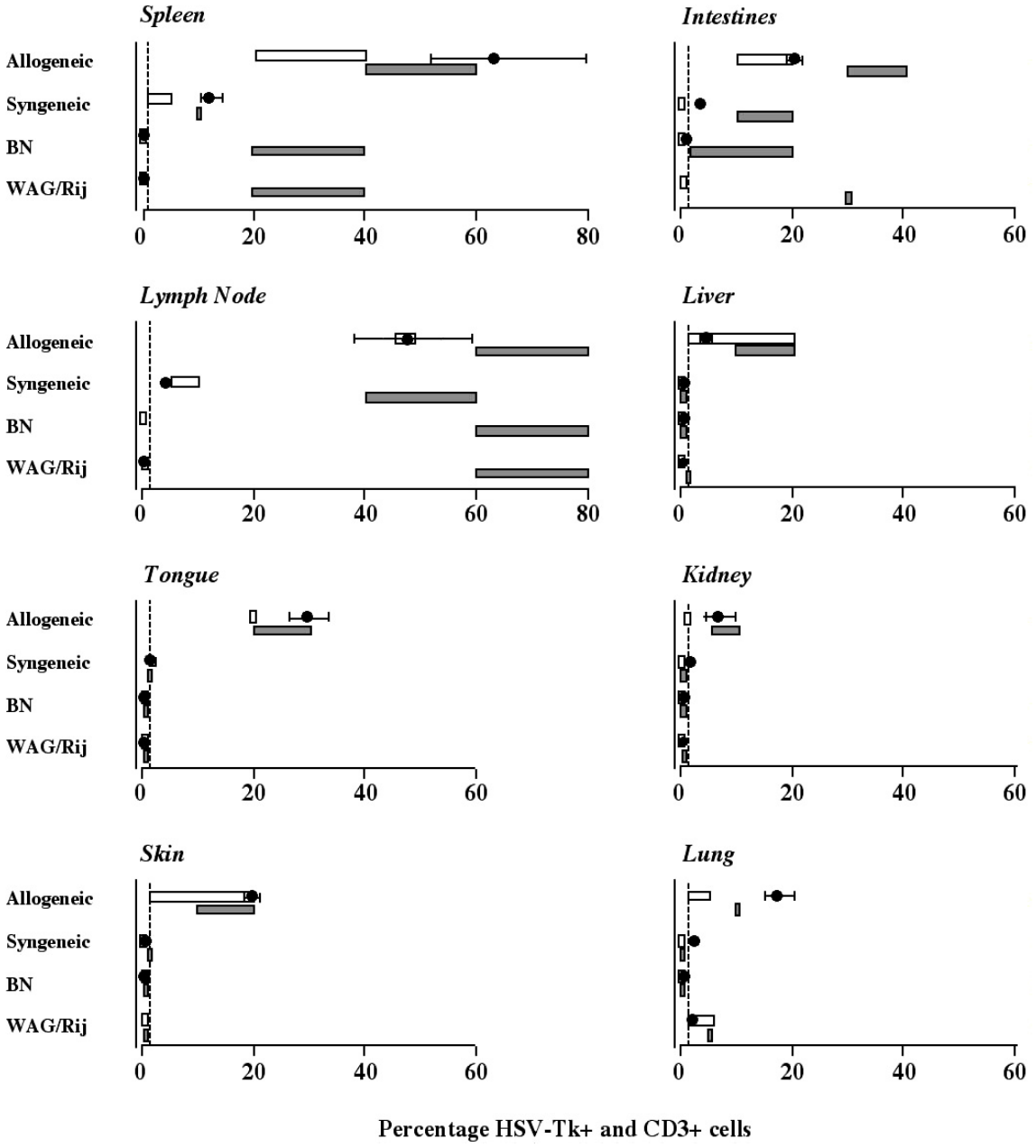

Figure 5. *Percentage of T cells and HSV-Tk/NGFR T cells in various tissues of allogeneic and syngeneic transplanted rats 18 days after BMT, and untransplanted BN and WAG/Rij rats. Immunohistochemical data for T cells (gray bars) and HSV-Tk/NGFR T cells (open bars) and real-time PCR data for HSV-Tk/NGFR T cells (solid circles) are given. Immunohistochemical data are given for one representative rat per group; real-time PCR data represents percentages of the same rats as used for immunohistochemical staining ± SD. Dotted line delineates 1% positivity.*

Low percentages of (HSV-Tk$^+$) T cells could not be quantified accurately by immunohistochemical analysis. Notably, immunohistochemical data showed that T cells in the allogeneic transplanted group predominantly were HSV-Tk/NGFR-transduced T cells. Only in typical lymphoid organs, for example, spleen, lymph

nodes and in mucosa-associated lymphoid tissue (MALT; intestine) a concomitant high percentage of nontransduced T cells was found.

Taken together, these data show that in allogeneic transplanted rats there was an increased infiltration of HSV-Tk$^+$ T cells in all tissues examined, especially in spleen, lymph node, tongue, skin, and intestine. Furthermore, in these rats there is a concurrent increase of non-transduced T cells in lymphoid and 'lymphoid associated' tissues. Finally, we show that real-time PCR analysis provides additional information in terms of accurate quantification, especially of low percentages, of transduced cells in tissues.

DISCUSSION

In the present report we monitored the involvement of HSV-Tk-transduced cells in the development of GVHD. Extensive manipulation of cells that have undergone *ex vivo* gene transfer by retroviral transduction could potentially modify their immune repertoire and their activation status, thus affecting their *in vivo* survival and function[6,19]. We have shown that HSV-Tk-transduced T cells are capable of exerting alloreactive responses in a rat model in which T cell alloresponses were induced after allogeneic BMT. HSV-Tk alloreactivity was demonstrated in peripheral blood, where a sudden increase in the amount of allogeneic HSV-Tk$^+$ T cells, that was not observed in syngeneic HSV-Tk$^+$ T cells, coincided with the onset of progressive weight loss and closely preceded the development of clinical symptoms of GVHD. Furthermore, in all tissues examined higher percentages of allogeneic HSV-Tk$^+$ T cells were found in allogeneic animals than in syngeneic controls. This increase is likely to be the result of alloreactivity-induced proliferation, which is much more rapid than the increase seen in the syngeneic animals. In the latter situation the increase is probably a normal response to homeostatic signals and is in agreement with earlier observations by Maury *et al*., who used carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled T cells in a semi-allogeneic/syngeneic transgenic mouse model[20]. Notably, in GVHD target organs most T cells were HSV-Tk positive. In skin and tongue alloreactive T cells showed specific localization patterns in subepithelial regions, indicating specific homing to target sites.

Genetically modified T cells were quantified in blood and tissues by FACS, and by immunohistochemical and quantitative PCR analyses. This enabled a comparison of the utility of these techniques for monitoring alloreactivity of transduced cells *in vivo*. In general, the data obtained by these techniques correlated, and confirmed their validity. The use of immunohistochemical staining of tissues for quantification is in general difficult and could be done with an accuracy interval of only 1 to 20%. In contrast, genomic DNA samples for rapid real-time PCR analysis could easily be obtained, and quantification could be performed accurately, especially in samples with low percentages of HSV-Tk$^+$ T cells. Immunohistochemical staining, however, provided direct evidence of concentrated localization of alloreactive HSV-Tk$^+$ T cells in tissues undergoing a GVH reaction, that is, tongue, intestines, and skin. Also, the typical GVH-induced loss of tissue architecture was evident. This implies that HSV-Tk-transduced T cells indeed have retained their potential to induce a GVH reaction and presumably also a GVL reaction. However, quantification of low percentages of transduced T cells was complicated by low levels of false positive staining cells

among the controls (Fig. 5). FACS analysis was performed on peripheral blood, BM, and tissues from which suspensions could be made (lymph node and spleen). Notably, with FACS analysis of cell suspensions, as with immunohistochemical quantification, problems were encountered concerning GVH-induced damage of cells. Furthermore, FACS analysis required the coexpression of an additional target protein sequence, preferably on the cell surface. Paquin *et al.* [21] have described the use of a chimeric green fluoresecent protein (GFP)-HSV-Tk transgene that can serve as a bifunctional suicide and reporter transgene, circumventing the need for a specific mAb and concomittant coexpression.

Finally, detection and quantification of less than 1% transduced cells by either flow cytometry or by histochemical analysis is problematic. Maddens *et al.* [22] have described the use of a competitive PCR method for the quantification of HSV-Tk- and neomycin resistance gene-expressing cells. However, with this approach transduced target cells are required to contain either of the genes, whereas we designed primers and probes for Moloney murine leukemia virus (MoMLV)-specific DNA sequences. This enables the detection of cells marked with any MoMLV-derived retroviral vector, in contrast to the use of marker gene-specific sequences to detect and quantify labeled cells[23-26]. Furthermore, competitive PCR requires total DNA quantification by UV spectrophotometry, and is not totally accurate. Taken together, we have shown that real-time PCR enables fast, accurate, sensitive, and specific quantification of HSV-Tk-transduced T cells in both blood and tissues.

Our data show that the involvement (quantity and biodistribution) of HSV-Tk$^+$ T cells in *in vivo* alloreactivity can thus accurately be monitored. Animal models provide the means for extensive monitoring of *in vivo* survival and dissemination of adoptively transferred genetically engineered cells and to ascertain cause-effect relationships. In a currently ongoing study, using the same allogeneic rat transplantation model described here, we are monitoring the fate of HSV-Tk-transduced T cells while a developing GVHD is being controlled by treatment with ganciclovir. In human clinical studies HSV-Tk-transduced T cells have been used in the treatment of GVHD/modulation of GVH alloresponses. Bonini *et al.* showed that GVH reactivity could develop in patients who received a donor lymphocyte infusion (DLI) for the treatment of relapse after a previous bone marrow transplant[8]. They showed that GVHD could effectively be controlled by GCV treatment. However, there is no direct proof that the GVH reactivity was predominantly caused by the transduced T cells and that the GCV was responsible for the GVHD control. Tiberghien *et al.* applied suicide gene therapy using low numbers of HSV-Tk-expressing T cells soon after HLA-identical BMT. They found no acute toxicity, the gene-transduced cells persisted in circulation, and they observed GCV-sensitive T cell alloreactivity[27]. Our approach enables a more detailed insight in the fate and behaviour of infused labeled T cells, using peripheral blood samples and (small) tissue biopsies[28].
Beside genetic engineering of allogeneic lymphocytes, there is an increased focus on the modification of autologous lymphocytes and (other) immune-competent cells in the treatment of, for example, autoimmunity[29,30], acquired immune deficiency syndrome (AIDS)[31,32], and cancer[33,34]. Although a variety of transgenes are used in these studies, most of the vectors used for transduction contain a MoMLV backbone. This makes our PCR procedure widely applicable for the detection and quantification of transduced cells.

In summary, we show that the amount of genetically engineered cells and their distribution can be monitored by a variety of methods, each with its own specific advantages and disadvantages. The PCR-based method is most sensitive and specific and also most widely applicable. This enables study of the involvement of labeled cells in *in vivo* alloreactivity, the response of HSV-Tk-transduced T cells to GCV-induced suicide therapy, and specific dose-effect relationships of genetically engineered lymphocytes in a broad range of preclinical and clinical studies.

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CHAPTER 4

Rejection of donor lymphocytes after infusion in recipients that reverted to autologous hematopoiesis after bone marrow transplantation

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ABSTRACT

Donor lymphocyte infusion (DLI) can induce complete remission in patients with leukemia who relapsed after allogeneic stem cell transplantation (SCT). Lymphocyte chimerism of patients at the time of infusion might be related to response to this form of immunotherapy. Here, we studied clearance of donor T cells that were infused after SCT, using a well-established rat bone marrow transplantation (BMT) model. Rats receiving reduced intensity conditioning (RIC) transiently repopulated with donor hematopoietic cells after BMT. Thereafter, hematopoiesis reverted to full host origin within seven weeks. Survival time of retrovirally marked donor T cells after infusion in these rats was compared to survival time of infused T cells in recipients that showed stable donor hematopoiesis after standard intensity conditioning (SIC). Infused donor T cells were completely cleared in rats engrafted with host-derived hematopoietic cells after transient engraftment with donor cells. In contrast, significant numbers of infused donor T cells survived in rats that developed durable donor hematopoiesis. The rapid elimination of transferred donor T cells in rats repopulated with hematopoietic cells of host origin suggests that this clearance is an active process mediated by recipient T cells, likely sensitized in vivo. Furthermore, this study clarifies a mechanism that may contribute to unresponsiveness to DLI in relapsed patients with hematopoiesis that reverted to host origin after SCT.

INTRODUCTION

ALLOGENEIC STEM CELL TRANSPLANTATION (SCT) can efficiently cure leukemia patients. However, the number of patients who relapse after SCT is significant. Relapsed patients can be successfully treated by donor lymphocyte infusions (DLI)[1-3]. However, a significant percentage of patients fail to respond to this therapy. Recently, it has been shown that conversion to complete donor chimerism by DLI diminish the relapse rate[4-6]. Childs *et al*., described that patients treated for solid tumors with SCT after a non-myeloablative conditioning or reduced intensity conditioning (RIC) only show tumor remission after hematopoietic conversion to full donor chimerism[7]. Previously, we observed that a high percentage of T cells of host origin in the peripheral blood of relapsed patients at the time of DLI significantly correlates with non-responsiveness[8]. Absence of graft-versus-host disease (GVHD) in these patients suggests that infused donor lymphocytes were reactive neither to leukemia cells nor to normal tissues. Recently, Blazar *et al*. showed that host T cells inhibit the development of GVHD induced by DLI using a murine bone marrow transplantation (BMT) model[9]. However, donor-derived regulatory T cells developing post-BMT may

also be involved in suppression of GVHD after DLI, probably by induction of tolerance[10]. The mechanisms by which either donor-derived or recipient-derived T cells contribute to tolerance or non-responsiveness of infused donor lymphocytes are not clearly demonstrated yet. We hypothesized that recurrent T cells of recipient origin, present at the time of DLI, inhibit alloreactivity of infused donor lymphocytes by eliminating these cells[11]. To study this hypothesis we used an allogeneic BMT model in rats. BN rats received bone marrow of WAG/Rij rats together with a RIC regimen. BMT recipients initially engrafted with considerable numbers of donor-derived white blood cells (WBC) followed by engraftment with host WBC. Engraftment was accompanied with severe GVHD resulting in survival of approximately 30% of the rats. Host WBC recurred in surviving rats followed by complete host hematopoiesis.

In this study, we questioned the potential of infused donor T cells to achieve an immune response in rats with recurrent host hematopoiesis after BMT. Therefore, we infused retrovirally marked donor T cells and compared the percentage of donor T cells that survive in blood and tissues after infusion in rats repopulated with either recurrent host or persistent donor WBC. The results show that within 3 days, infused T cells are rejected in rats with recurrent host hematopoiesis after BMT. In contrast, significant numbers of retrovirally marked donor T cells persist after infusion in recipients that developed stable donor hematopoiesis after BMT.

MATERIALS AND METHODS

BMT and DLI

BN rats (BN/RijHsd, RT-1A^n) and WAG/Rij rats (WAG/RijHsd, RT-1A^u) were transplanted with bone marrow cells of WAG/Rij rats. Rats were obtained from Harlan (Horst, The Netherlands) and kept in filter top cages and given sterilized food and acidified water. The RIC or non-myeloablative conditioned group (n=17) received an intra peritoneal injection of 1 ml rabbit anti-rat lymphocyte serum (Sanbio BV, Uden, The Netherlands) 5 days before BMT. In addition, tacrolimus (FK506, 1 mg/kg, Fujisawa GmbH, München, Germany) was intramuscular injected daily, from day –1 until day +10 of BMT, and total body irradiation (TBI) with low dose (4.3 Gy) was given 6 hours before BMT. SIC or myeloablative conditioned rats received high dose TBI (7.2 Gy) without further treatment before allogeneic (n=5) or syngeneic (n=5) transplantation. Marrow cells of WAG/Rij donors were collected by flushing femurs and tibiae with RPMI (Gibco BRL). All rats received 5×10^7 WAG/Rij bone marrow cells via injection into the tail vein.

RIC treated BN rats received DLI after they recovered from GVHD and developed full host hematopoiesis (day 49 post BMT). SIC treated BN and WAG/Rij rats repopulated with WAG/Rij blood cells were infused 21 days after BMT. All transplanted rats and 4 untreated BN rats received DLI with 2×10^7 retrovirally marked WAG/Rij T cells via injection into the tail vein.

Retroviral transduction of WAG/Rij splenic lymphocytes

LZRS-TN, a MoMLV-based retroviral vector comprising the SFCMM-3-derived Herpes Simplex Virus (HSV)-1 thymidine kinase (Tk) gene, the SV40 promoter and the truncated human nerve growth factor receptor (NGFR)[12], cloned into the LZRS vector[13], was used to transduce WAG/Rij splenic T cells as described previously[14]. Cells expressing NGFR were purified (purity >95%) 4 days after ConA (5 mg/ml) activation as described[14], and infused 1 day later.

Flow cytometry

Rat MHC haplotype of peripheral WBC was analyzed by flow cytometry (Epics XL, Beckman Coulter, Fullerton, CA, U.S.A.) using U9F4 and OX27-FITC mAb to determine WAG/Rij and BN origin, respectively. RPE-conjugated $F(ab')_2$ fragments of goat anti-mouse IgM (DAKO, Glostrup, Denmark) were added for detection of U9F4-positive cells. The percentage transduced cells was determined by flow cytometry after staining with NGFR specific mAb 20.4. T cells were measured by flow cytometry using fluorescent conjugated mAb directed against αβ-TCR, CD4 and CD8 (R73, W3/25 and OX-8, respectively; Immunotech, Marseille, France).

DNA isolation and real-time quantitative PCR

Whole blood was collected from the tail vein into sodium citrate (3.8%; v/v 10:1). Cell samples were pre-treated with dextran to remove erythrocytes. DNA from WBC and transduced T cells was isolated using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany). DNA was isolated from representative specimens of spleen, liver and lungs, and from cell suspensions of pooled lymph nodes, using the QIAamp DNA Mini Kit (Qiagen). The number of transduced T cells in peripheral blood and various organs after DLI was quantified by real-time PCR analysis using the 5' nuclease assay (Taqman) and the ABI/PRISM 7700 sequence detector (Perkin-Elmer Applied Biosystems, Fostercity, CA, U.S.A.) as described previously[15]. DNA of purified transduced T cells (95% pure) was used to determine amplification efficiencies. Genomic DNA was serially diluted in dH2O, ranging from 750 ng to 75 pg. The proviral amplicon as well as the rat preproalbumin locus were amplified. Initial template concentrations were related to cycle threshold (C_t). The difference in C_t (ΔC_t) for each dilution was constant over a dynamic template concentration range of 5 logs, demonstrating that amplification is equally efficient in both reactions, resulting in detection threshold of 0.01% labeled cells.

RESULTS

Analysis of GVHD and WBC chimerism after allogeneic BMT

Body weight (BW) of the animals was recorded after BMT as parameter to measure the onset and progression of GVH reactivity. BN rats that received the RIC regimen recovered from the BMT procedure from day 5 onward, as shown by increase of BW. At day 23 after BMT, BW of all rats decreased rapidly, which coincided with other symptoms of severe GVHD. Sequentially, the majority of rats died of GVHD or

became moribund and therefore killed (< 34 days after BMT). Thereafter, surviving rats showed rapid increase of BW after day 34, which indicated recovery from GVHD. Figure 1 shows BW of surviving rats. BW of rats that did not survive was within the range of BW of surviving rats (data not shown). In contrast, BN rats that received standard conditioning regained normal BW within 21 days after BMT after transient weight loss due to the transplantation procedure (data not shown).

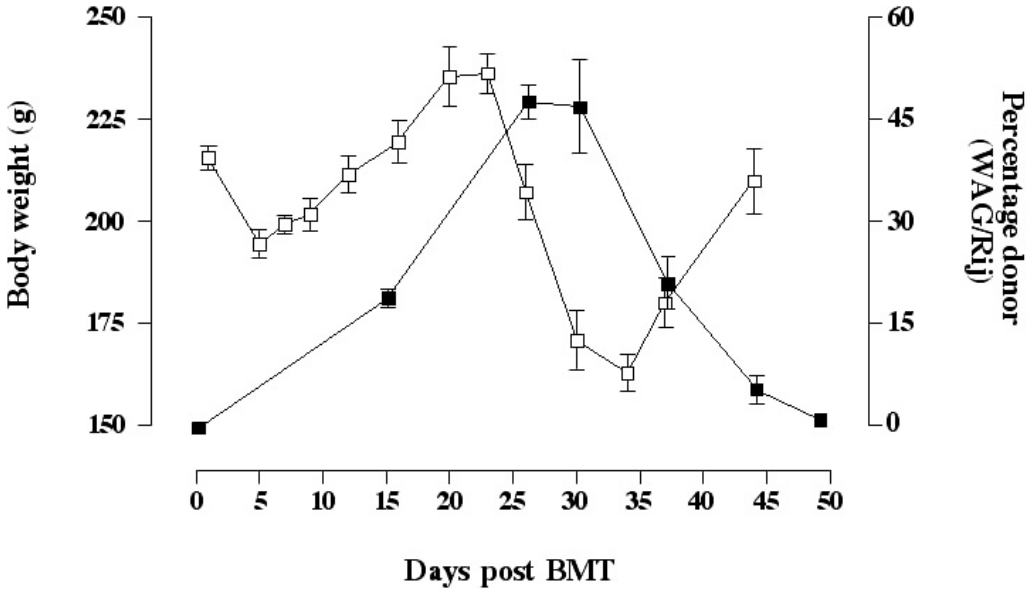

Figure 1. *BW and WBC chimerism after BMT with RIC regime. BW (□) of animals that received BMT with a RIC regimen dropped between day 23-26 demonstrating development of severe GVHD. Percentage of donor WBC WAG/Rij (■) increased after BMT and peaked at day 26. Thereafter, all rats reverted to autologous hematopoiesis Severity of GVHD showed coincidence with peak levels of donor WBC. Reversion to host hematopoiesis in surviving rats was followed by recovery of GVHD, shown by increase of BW. Data represents mean values for surviving rats (n=5) ± SEM.*

The genetic origin of WBC repopulated in rats that received allogeneic BMT was analyzed. Rats treated with the RIC regimen prior BMT showed an increase of the percentage of donor WBC until day 26 after BMT (Figure 1). Thereafter, hematopoiesis of all surviving rats reverted to host origin (complete at day 49). WBC of BN rats that received the standard conditioning regimen converted to donor origin, ranging from 94% to 97%, within 16 days (data not shown).

These data demonstrate that allogeneic BMT with RIC results in development of severe GVHD. Rats show reversion of donor hematopoiesis to host hematopoiesis concurrent with GVHD. In contrast, SIC before BMT results in stable donor hematopoietic reconstitution without symptoms of GVHD. These observations suggest that host hematopoietic cells that survived the conditioning regime can enhance the occurrence and severity of GVHD. Disappearance of donor-derived alloreactive cells seems required to overcome GVHD resulting in survival of the rats. Recurrent host-derived WBC are probably responsibly for disappearance of these alloreactive donor WBC.

Analysis of T cell numbers in peripheral blood after DLI.

Rats received DLI after transplantation with WAG/Rij bone marrow cells. DLI was given to rats at the time that BW reached pre BMT levels. Therefore, BN rats treated with RIC and BMT received DLI at day 47, *i.e.* at the time they recovered from GVHD. BN rats and WAG/Rij rats treated with SIC and BMT, and untreated BN received DLI at day 21. All rats were infused with 2×10^7 marked WAG/Rij T cells. At the time of DLI, the number of T cells in BN rats that reverted to host origin after allogeneic BMT with RIC was hardly lower than T cell numbers in untreated BN rats (2.2×10^6/ml, versus 4.2×10^6/ml). Allogeneic transplanted BN rats repopulated with WAG/Rij T cells, showed numbers of T cells than syngeneic transplanted WAG/Rij at the time of DLI (0.6×10^6/ml versus 0.7×10^6/ml). T cell numbers in rats with WAG/Rij hematopoiesis was lower than in rats with BN hematopoiesis. However, T cell numbers reflected the amount of T cells in untreated WAG/Rij and BN rats.

After DLI the total amount of T cells in the peripheral blood of rats was monitored. T numbers cells slightly increased after DLI in rats that reverted to host origin after allogeneic BMT (Figure 2A). T cell numbers in rats treated with allogeneic BMT and SIC did not change after DLI (Figure 2A). In addition, syngeneic transplanted rats and rats untreated before DLI showed stable T cell numbers after DLI (Figure 2B).

These data show that at the time of DLI, T cell restitution in rats that recovered from severe GVHD after BMT with RIC is comparable with that in rats that did not develop GVHD after BMT with SIC. Furthermore, the data show that DLI results in a slight and transient increase of T cell numbers in BN rats with WBC that reverted to autologous hematopoiesis after BMT, and do not affect T cell numbers in other groups of rats treated with DLI.

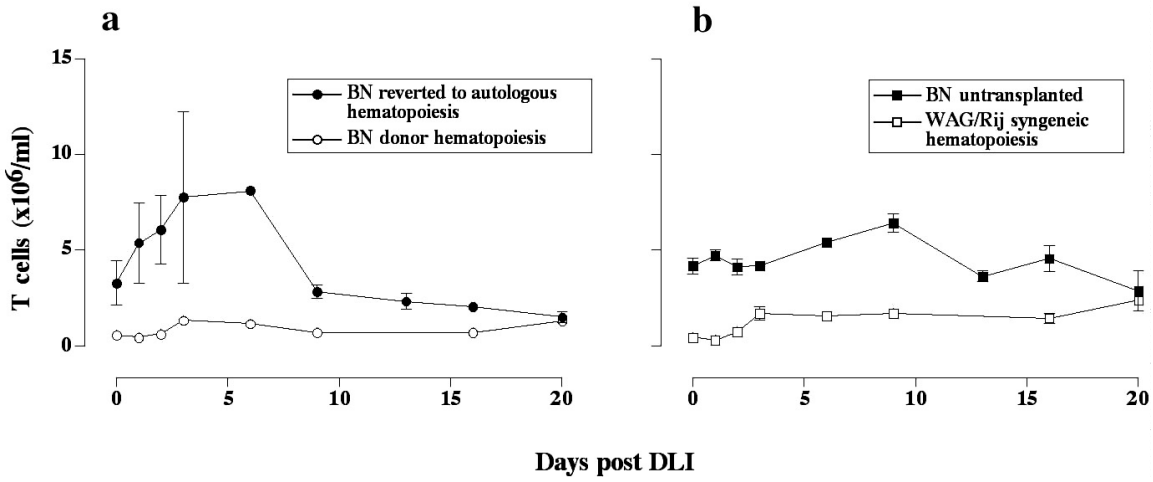

Figure 2. *T cell counts after DLI. The percentage of TCR positive cells was measured by flow cytometry. Numbers of T cells per ml blood were calculated from WBC numbers. (A), BN rats that reverted to autologous hematopoiesis after transient mixed chimerism (●) showed a slight increase of T cells after DLI. T cell numbers in BN rats with stable donor hematopoiesis did not change after DLI (○). (B) T cell numbers in syngeneic WAG/Rij transplanted rats (□), and BN rats that received no treatment for DLI (■) are stable after infusion. All rats received 2×10^7 marked WAG/Rij T cells. Data represents mean values ± SEM of 5 rats except the BN nontransplanted rats (n=4).*

In vivo survival of infused T cells in peripheral blood

Retrovirally labeled WAG/Rij T cells (2×10^7) were infused to determine in vivo survival after injection. BN rats that reverted to autologous hematopoiesis (BN) showed rapid clearance of infused labeled WAG/Rij T cells in blood (Figure 3A). Within 24 hours, the amount of infused T cells declined from 60×10^3/ml (4 hours post DLI) to 20×10^3/ml and reached limit of detection (<100 cells/ml) within 2 days. In contrast, BN rats with stable WAG/Rij hematopoiesis after BMT tolerated infused labeled donor T cells (Figure 3A). WAG/Rij T cells persisted during the follow up period (20 days). Comparable amounts of labeled T cells were found in blood of syngeneic transplanted WAG/Rij rats after infusion (Figure 3B). Untreated BN rats rapidly rejected labeled WAG/Rij T cells after infusion (Figure 3B). The kinetics of WAG/Rij cell clearance in allogeneic transplanted BN rats that reverted to recipient WBC origin showed similar kinetics than that observed in non-transplanted BN rats. This suggests that clearance of infused WAG/Rij T cells is an active process in both untreated BN rats and BN rats with recurrent autologous hematopoiesis after BMT.

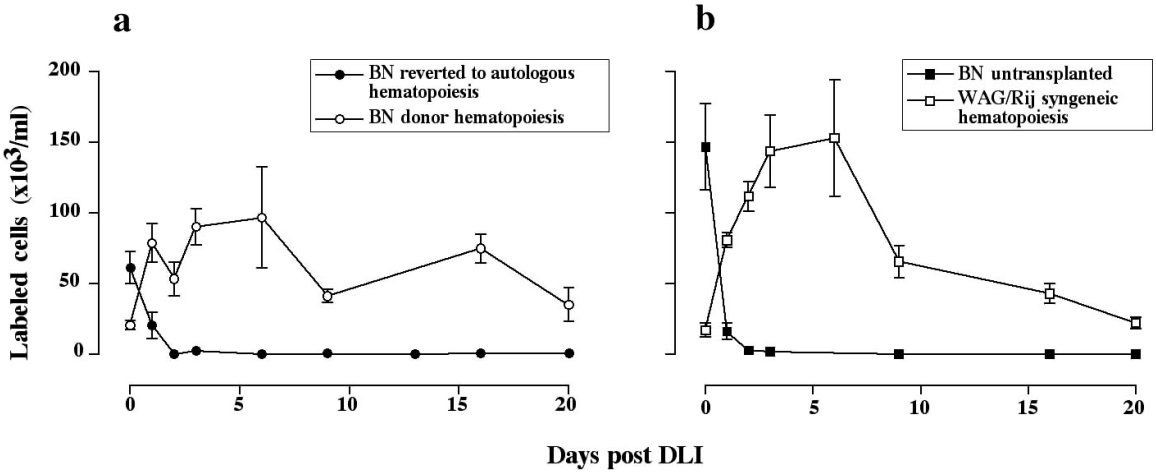

Figure 3. *Survival of infused T cells in peripheral blood. The amount of retrovirally labeled WAG/Rij T cells was measured at regular intervals after infusion. (A) Marked WAG/Rij T cells rapidly decline after infusion in BN rats that reverted to autologous hematopoiesis after transient mixed chimerism (●) in contrast to marked T cells in BN rats that had developed stable donor hematopoiesis (○); (B) Marked WAG/Rij T cells survive partially in syngeneic transplanted WAG/Rij rats (□) and disappeared rapidly in BN rats that received no previous treatment (■). All rats received 2×10^7 marked WAG/Rij T cells. Numbers of labeled T cells per ml blood were calculated from WBC numbers. Data represents mean values ± SEM of 5 rats except the BN non-transplanted rats (n=4).*

In vivo survival of infused T cells in tissues

Rats were killed 20 days after DLI to study the persistence of donor T cells after infused in a number of organs. DNA was isolated from representative specimens of spleen, liver and lung, and cell suspensions of pooled lymph nodes. Figure 4 shows that all examined organs of allogeneic transplanted BN rats with hematopoiesis that reverted from donor to host after BMT did not contain labeled T cells above detection limit (< 0.01%). In contrast, significant percentages of labeled donor T cells were present in the organs of BN rats that repopulated with donor-derived hematopoietic cells after BMT (Figure 4). The percentages labeled cells measured in the organs of

these animals were comparable or even higher than found in the organs of syngeneic transplanted WAG/Rij rats (Figure 4). Labeled WAG/Rij T cells were not detectable in untreated BN rats 20 days after infusion (Figure 4).

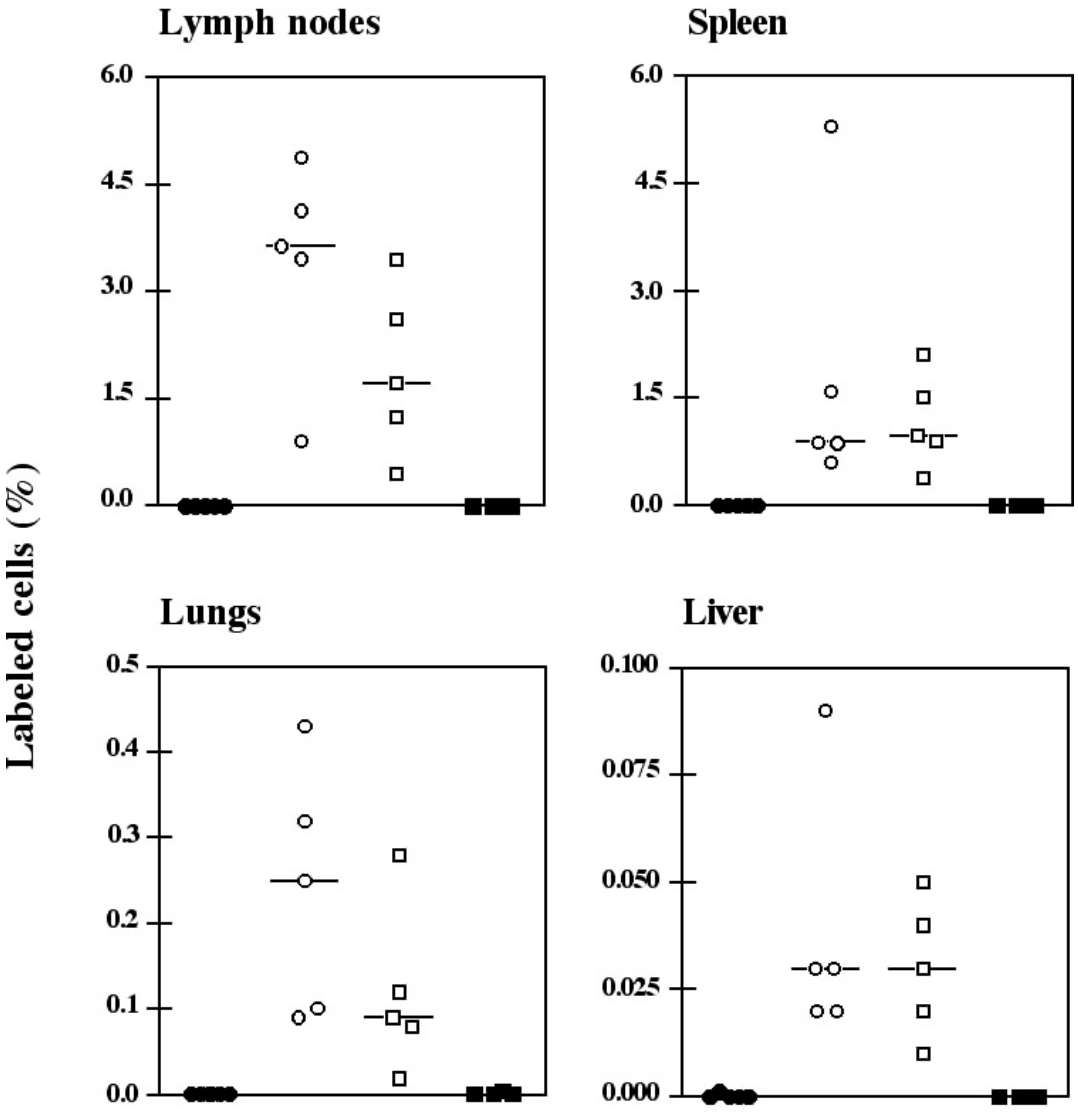

Figure 4. *Survival of infused T cells in tissues. The percentage of labeled cells in spleen, lymph nodes, liver and lungs at day 20 post DLI was determined. Infused marked WAG/Rij T cells could not be detected in tissues of BN rats that reverted to autologous hematopoiesis (●) and in nontransplanted BN rats (■) (threshold of detection = 0.01%). Significant percentages infused marked WAG/Rij T cells survive in rats that repopulated with WAG/Rij hematopoietic cells after BMT (○) and syngeneic transplanted rats (□). All rats received 2×10^7 marked WAG/Rij T cells. Data represents mean values ± SEM of 5 rats except the BN untransplanted rats (n=4). All groups consisted of 5 rats, except the BN non-transplanted rats (n=4). The percentage of LZRS-TN transduced cells was determined by reference to a calibrator DNA sample of purified HSV-Tk/NGFR transduced T cells (purity ≥ 95%) after a real-time PCR analysis, using the 5' nuclease assay (Taqman) and the ABI/PRISM 7700 sequence detector. Numbers of labeled T cells per ml blood were calculated from WBC numbers. Data represents mean values ± SEM.*

These observations show that labeled donor T cells infused after BMT migrate to tissues. Moreover, we observed a remarkable difference in the number of T cells surviving in the tissues of DLI recipients with either BN or WAG/Rij hematopoiesis. The absence of marked donor lymphocytes in transplanted rats with hematopoietic cells that reverted to recipient 20 days after infusion suggests an active elimination of these cells, likely exerted by in vivo primed recipient-derived T cells.

DISCUSSION

In this study we show that infused donor T cells are rapidly removed in rats that regain host hematopoiesis after a transient engraftment with donor cells. The fast kinetics of disappearance in rats repopulated with host WBC and the much slower disappearance in rats repopulated with donor WBC suggest that alloreactive host T cells reject infused donor cells. Recently, Blazar *et al*. have demonstrated in a mouse model that host T cells can suppress alloreactivity induced by DLI. They suggest that host T cells are capable of generating anti donor cytotoxic activity resulting in an impaired ability of DLI to induce GVHD, likely due to elimination of infused donor cells[9]. Strong alloreactivity resulting in rapid elimination of allogeneic lymphoid cells after infusion in untreated mice has been previously described[16,17] and is confirmed by our findings in untreated BN rats that received lymphocytes of WAG/Rij rats. Rejection of infused donor T cells in rats engrafted with host T cells, as we have clearly shown here, may reveal one of the mechanisms that limit the efficacy of DLI in cancer therapy.

We observed in our rat model that recurrence of host hematopoiesis after BMT coincide with the onset of severe GVHD. In contrast, no sign of GVHD was observed in rats that developed full donor hematopoiesis after BMT with SIC. These observations suggest that recurrent host hematopoietic cells may activate donor T cells and thereby induce severe GVHD. A prominent role of host antigen presenting cells (APC) in inducing GVHD has been demonstrated previously[18]. Mapara *et al*. showed that host APC can enhance alloreactivity directed against leukemia cells[19]. Moreover, Shlomchik *et al*. showed that inactive host APC (MHC class I negative) present after BMT prevent development of GVHD. Although MHC class I positive donor-derived APC are present at that time these cells could not induce GVHD, showing that host APC are responsible for initiating GVHD[20]. Auffermann-Gretzinger *et al*. showed that blood dendritic cells (DC) of host origin are present during a short period after SCT in humans. The majority of DC (80%) is of donor origin 14 days after SCT[21]. However, preterminal host DC present in irradiated mice during a short period after BMT can efficiently trigger donor T cells before these DC disappear as shown by induction of GVHD[22].

Rats receiving RIC and BMT in our transplantation model, suffered severely of GVHD and rats that survived GVHD showed conversion of hematopoiesis from mixed to full host origin. This conversion started coincidently with morbidity and mortality of GVHD. These observations suggest that strong alloreactivity can occur during hematopoietic chimerism and may be exerted by both donor and host lymphocytes. The results of this two-way alloreactivity may be contrary. Alloreactivity that is dominated by donor-derived T cells may result in death of rats by GVHD. Alloreactivity that becomes dominated by host-derived T cells may

eliminate donor cells including alloreactive T cells and thereby overcome GVHD. Successively, these allogeneic activated host T cells may be responsible for rejection of labeled donor T cells after infusion.

We demonstrated in our rat model that MHC class I alloreactive host T cells eliminate donor cells and thereby reduce the success of DLI after BMT. The majority of human have been transplanted with HLA identical stem cells. However, disparities in minor histocompatibility antigens (mHag) can also initiate strong alloreactivity[23,24]. Host cytotoxic T cells specificly directed against mHag have been isolated from patients after rejection of HLA-identical stem cell-grafts[23,24]. Moreover, Vogt *et al*. have shown that sensitization of female recipients prior to SCT by blood transfusion of male donors increases the number of specific T cells directed against the H-Y mHag. The presence of H-Y antigen specific T cells resulted in an enhanced frequency of graft rejection[25].

In summary, we show in allogeneic rat model that reversion of hematopoiesis from donor to host after SCT results in alloreactivity against donor cells. Alloreactive host T cells eliminate infused donor T cells. This mechanism can contribute to failure of adoptive allogeneic immunotherapy after SCT in humans who show hematopoietic cell reversion from donor to host origin.

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CHAPTER 5

Quantification of donor and recipient hemopoietic cells by real-time PCR of single nucleotide polymorphisms

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ABSTRACT

Analysis of changes in recipient and donor hemopoietic cell origin is extremely useful to monitor the effect of stem cell transplantation (SCT) and sequential adoptive immunotherapy by donor lymphocyte infusions (DLI). We developed a sensitive and accurate method to quantify the percentage of recipient and donor cells by real-time PCR using single nucleotide polymorphisms (SNPs) as markers. Allele specific PCR of seven SNPs resulted in specific markers for donor or recipient in 97% of HLA-identical sibling pairs. Both, recipient- and donor-derived hemopoietic cells can be simultaneously analyzed in 67% sibling pairs. We expect this can be increased to approximately 99% by developing three additional SNP-PCR. Serial dilution of SNP-positive DNA into either SNP-negative DNA or water revealed a detection limit of 0.1-0.01% depending on the amount of input DNA and start C_t of the used SNP-PCR. Application of our real-time SNP-PCR method for a CML patient treated with allogeneic SCT and DLI demonstrated its feasibility to follow donor T cell chimerism and early detection of residual and recurrent autologous hemopoiesis in response to treatment. This detailed monitoring of the genetic origin of hemopoietic cells, in particular immune effector cells and target cells after SCT and DLI, may substantially contribute to understanding of the mechanisms that play a role in the success of treatment.

INTRODUCTION

TRANSPLANTATION WITH HEMATOPOIETIC STEM CELLS from HLA-identical sibling donors has been successfully used to treat patients with hemopoietic malignancies. Allogeneic stem cell transplantation (SCT) results in effective replacement of eradicated recipient stem cells. Moreover, immunereactivity of donor effector cells against residual malignant cells contributes significantly to the success of treatment. This immunoreactivity of donor T cells also limits the success rate of the therapy by transplantation-related mortality because of severe graft-versus-host-disease (GVHD)[1]. T cell depletion of the stem cell graft reduces the incidence and severity of GVHD but results in an increase of relapse rate, which confirms the contribution of donor derived immunocompetent T cells in eliminating residual malignant cells[2]. In addition, the significantly higher relapse rates in patients who received stem cells of genetically identical twins compared to patients who received stem cells of HLA-identical siblings strongly supports the hypothesis that minor alloantigens significantly contribute to the induction of immunereactivity of donor lymphocytes against malignant cells[3]. Attempts are made to further exploit the immunereactivity of donor cells against recipient hemopoietic cells, including

leukemia and lymphoma cells using a nonmyeloablative conditioning regime prior transplantation, followed by donor lymphocyte infusion (DLI)[4,5]. Interestingly, this treatment regime has also been applied to treat solid tumors and revealed that achievement of full T cell chimerism was a prerequisite for response to treatment[6-9].

DLI can be very effective in preventing and curing relapse of leukemia but harbors the risk of inducing fatal GVHD[10-13]. Although the response rate of patients, treated for CML, to DLI is quite high, the majority of patients with acute myeloid or lymphoid leukemia (AML and ALL) do not respond to DLI[14]. Success of DLI may be improved when dose and timing of given donor T cell infusion can be adapted to each individual patient, thereby minimizing the risk of GVHD and maximizing the reactivity against malignant cells[15]. Mackinnon *et al.*[16] have shown that repetitive administration of increasing numbers of donor lymphocytes resulted in complete remission in patients with CML who did not respond to low-dose DLI. Moreover, infusions of escalating numbers of donor T cells induced less GVHD compared to DLI given as bulk dose[17]. In addition, *in vitro* activation of donor cells can improve DLI. Slavin *et al.* showed[18] that recurrent leukemia cells in patients not responding to DLI could be effectively eliminated by donor T cells activated by IL-2 *ex vivo*. Furthermore, early detection of relapse may enhance the success rate of DLI by treatment before the onset of overt clinical relapse[19]. Increasing numbers of BCR-ABL-expressing cells can indicate early relapse of CML[10-22]. Other fusion transcripts can be indicative for relapse of acute leukemia patients[23,24]. It has also been suggested that monitoring of chimerism in lymphoid and myeloid subsets, isolated from peripheral blood of patients after SCT, allows detection of residual or recurrent leukemia cells[25-27]. Imminent relapse of leukemia lacking a genetic marker may be prevented by adoptive cellular immunotherapy given early after an increase of percentage of recipient cells[26].

The mechanisms responsible for failure of immunotherapy after SCT are not clear. A high percentage of autologous T cells coincided with reduced alloreactivity of infused donor T cells, which may suggest rejection of infused T cells or induced T cell tolerance[28,29]. Donor-derived regulatory T cells may also suppress reactivity of infused donor lymphocytes[30]. Frequent analysis of chimerism in lymphoid and myeloid subsets after SCT may be of great value to identify patients with high risk for relapse or graft rejection[24,25,27,31]. Several techniques have been used in these studies to monitor chimerism after SCT. PCR of DNA sequences with tandem repeats (VNTR, STR) or satellite DNA has applied frequently[25-27]. FISH analysis to discriminate male and female cells have been utilized in sex-mismatched sibling pairs.

We have developed a real-time PCR method based on the detection of biallelic single nucleotide polymorphisms (SNPs). Biallelic SNPs exist with a very high frequency in the human genome[32]. We show that the method is applicable for almost all recipient/donor pairs and can accurately quantify at least 0.1% recipient cells among donor cells and *vice versa*. Detailed monitoring of the genetic origin of hemopoietic cells after SCT and DLI can substantially contribute to understanding of the mechanisms involved in response to therapy and guide adoptive immunotherapy strategies.

MATERIAL AND METHODS

Patient and cell samples

Chimerism of hemopoietic cells was studied in patients who received T cell-depleted SCT with stem cells from a HLA-identical sibling donor. CML patient (UPN 480) with Philadelphia chromosome positive (Ph+) CML cells was conditioned with 120 mg cyclophosphamide per kg body weight and total body irradiation (9 Gy). Cyclosporin A was given until two months after SCT to prevent GVHD. PBMC from peripheral blood collected before and following SCT and DLI were isolated by Ficoll density gradient centrifugation. T cells and myeloid cells were isolated by flow cytometry (Epics Elite, Beckman Coulter, Fullerton, CA, USA) after staining with CD3-FITC-conjugated mAb (UCHT1 Beckman Coulter) or CD13/CD33-PE-conjugated mAb (WM-54, WM 47, respectively, Dako, Glostrup, Denmark). Purity of both cell populations was > 99.5%.

DNA preparation and SNP-typing by RLFP analysis

Genomic DNA was isolated with QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) from sorted cell populations, PBMC of donors and recipients collected before SCT, and from EBV-transformed B cells generated from PBMC of recipients collected prior SCT and donors (see the Appendix for detailed information). DNA fragments containing SNPs in *PECAM1*, *ICAM1, HA1*, *MLH1*, *SUR1*, and the sequence-tagged sites (STSs) G42863, and G42888, were amplified as described before[33-41]. Genotype of SNPs was determined after digestion with restriction enzyme *Pvu*II (*PECAM1* and *MLH1*) and *Pst*I (*SUR1*) by agarose gel electrophoresis[33,34,41]. Genotype of SNPs in *ICAM1, HA1*, G42863 and G42888 was determined by DNA sequencing of PCR products.

Specific amplification of SNPs by real-time PCR

DNA isolated from EBV-transformed cell lines bearing homozygously the identified SNP was used to develop and optimize Taqman-based real-time PCR for each of the SNPs (Perkin Elmer Applied Biosystems, ABI Prism 7700)[42-44]. In addition to allele-specific real-time PCR, we developed an SMCY-gene (accession number: AF273841) real-time PCR to quantify male cells (see the Appendix for detailed information).

Quantification of the percentage of cells containing allele-specific sequences

Calibration functions were generated from C_t obtained by real-time PCR of serially diluted DNA, isolated from cell samples harboring each of the SNPs homozygously, heterozygously, or hemizygously. These calibration curves were used to calculate the percentages of recipient and donor cells in the blood samples collected after SCT (see the Appendix for detailed information). The amount of input DNA was acurately defined by real-time PCR using a DNA fragment encoding albumin.

Calculation of the discriminating capacity of biallelic SNPs

Genotype frequencies obtained by SNP-PCR from of 80 SCT recipients and their HLA-identical sibling donors were compared with SNP frequencies described

earlier[33-41]. The genotype frequencies we obtained by real-time PCR of SNPs were used to analyze the discriminative capacity of the seven SNPs. Calculation of probability that siblings have different genotypes with alleles A and B that are located on autosomes revealed the following formula: [(Freq AA + 5/8 Freq AB + Freq BB) × Freq AB]. The probability that siblings have different genotypes of SNPs located on the X chromosome was calculated by the formula: [5/8 Freq AB + 1/2 Freq A × Freq BB + 1/2 Freq B Freq AA].

RESULTS

Development of SNP-specific real-time PCR for identification of recipient and donor cells

Seven SNPs of which high frequency in human populations have been described, were selected for the identification of recipient- and donor-derived cells after SCT (Table 1). SNPs located on chromosome 6, which contain the MHC complex encoding the HLA molecules, were excluded. RFLP analysis and DNA sequencing of amplified fragments were used to define cells bearing the selected SNPs, homozygously, heterozygously, or hemizygously. Using DNA isolated from these cells, we developed real-time PCR with SNP-allele-specific primers and gene-specific probes.

Table 1. Characteristics of SNPs used as specific markers for recipient and donor cells

Gene	*Chromosome*	*SNP*	*Location*	*Frequency (%)*[a] *AA/AB/BB*	**Reference**
PECAM1	17	C G	bp 514 codon 125	28/42/30	33, 34
ICAM1	19	G A	bp 1462 codon 214	20/47/33	35, 36
HA1	19	C-A T-G	bp 500-504	17/49/34	37, 38
MLH1	3	G A	promoter bp –93	26/55/19	39
SUR1	11	C T	exon 16 bp –3	32/47/20	40
STS[b]	*Chromosome*	*SNP*		*Frequency (%)*[c] *A/B*	**Reference**
G42863	Xq28	A C		68 32	41
G42888	Xq25	T C		60 40	41

[a] Genotype frequencies given by the literature.
[b] Sequence-tagged site.
[c] Allele frequencies given by the literature.

To hinder amplification of noncomplementary alleles, we synthesized SNP-allele-specific primers containing one extra mismatch in one of the two adjacent nucleotides of the polymorphic nucleotides. We replaced these nucleotides for one of the three alternative nucleotides and determined which of these mismatched primers resulted in highest specific amplification and lowest background amplification. We observed significant differences in amplification efficiency and target specificity of allele-specific primers as shown for the A allele of *MLH1* (Fig. 1). High fluorescence signal early after amplification of the positive allele (*MLH1*-A, C_t 24.5), and low fluorescence during amplification of the negative allele (*MLH1*-G, C_t 35.9), was observed using the allele-specific primer ending with ATT (primer set 1 in Figure 1). In contrast, allele-specific primer ending with TAT (primer set 2 in Figure 1) showed lower sensitivity for *MLH1*-A (C_t 28.2), and allele-specific primer ending with CTT (primer set 3 in Figure 1) showed high background amplification of the negative allele *MLH1*-G (C_t 29.1).

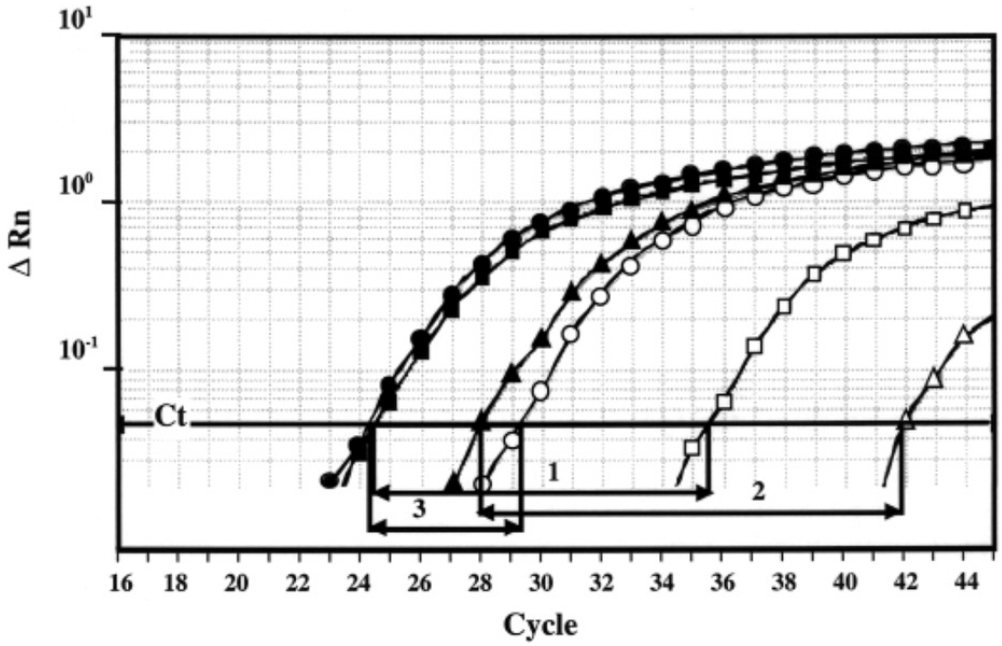

Figure 1. *Amplification curves of the MLH1 SNP-alleles by real-time PCR. Amplification plots of PCR using three reverse primers specific for the A allele and a common forward primer are shown. Primer set 1, with reverse primer 5'-TCGTGCTCACGTTCTTCCAT**T**-3' reached threshold after 24.5 cycles for the A allele* (■) *and after 35.9 cycles for the G allele* (□) *(ΔC_t =11.4). Primer set 2, with reverse primer 5'-TCGTGCTCACGTTCTTCCTA**T**-3' reached threshold after 28.2 cycles for the A allele* (▲) *and after 41.8 cycles for the G allele* (Δ) *(ΔC_t =13.6). Primer set 3, with reverse primer 5'-TCGTGCTCACGTTCTTCCCT**T**-3' reached threshold after 24.4 cycles for the A allele* (●) *and after 29.1 cycles for the G allele* (○) *(ΔC_t =4.7). Primer set 1 gave the best results for the MLH1-A allele.*

Using this approach we developed primer sets that reached the detection threshold before 25 cycles of amplification, and showed background amplification of the non-complementary allele after 35 cycles, using 500 ng of genomic DNA. Table 2 shows the developed primer sets, the optimum annealing temperature, and the amplification efficiency (C_t, and ΔR_n after 45 cycles of amplification), obtained by real-time PCR for seven SNPs. Cycle threshold of amplification of repeated experiments ranged

within 0.5 cycle, starting with the same amount of target DNA. Log dilutions of target DNA resulted in C_t differences of approximately 3 cycles. Theoretically, differences of more than 10 cycles in reaching the threshold between the positive and negative allele allowed quantification of at least 0.1% target DNA. Fixed anealing/elongation temperature of 60°C to screen 100 ng DNA for all seven SNPs in one real-time PCR run resulted in $C_t < 30$, using allele-specific primers for *ICAM1*, *HA1*, *MLH1*, and *SUR1*. Allele-specific primers for PECAM1 and G42863; G42888 were shortened to reach $C_t < 30$ after amplification of 100 ng DNA.

The results shown here demonstrate that real-time PCR of target sequences with SNPs can be used to identify the genetic origin of cells. Moreover, this method is highly reproducible and applicable for the detection of very small percentages of cells with specific SNP-markers.

Table 2. Amplification characteristics of developed SNP allele-specific primers for real-time PCR

Gene/STR	*SNP*	*Alle-specific primer (5' to 3')*[a]	*Amplification*				ΔC_t[c]
			pos. allele		*neg. allele*		
			ΔC_t	ΔR_n[b]	C_t	ΔR_n[b]	
PECAM1	C	AGGACTCACCTTCCACCAACCC**G** (R)	22.9	1.1	44.8	0.1	21.9
	G	AGGACTCACCTTCCACCAACCT**C** (R)	25.0	1.1	41.4	0.1	16.4
ICAM1	G	AGAGCACATTCACGGTCACCCA**C** (R)	25.5	1.1	38.1	0.3	12.6
	A	AGAGCACATTCACGGTCACCA**TT** (R)	24.9	1.1	36.1	0.8	11.2
HA1	C-A	GCTCTCACCGTCA**C**GCA**A** (R)	24.9	1.4	39.9	0.1	15.0
	T-G	GCTCTCACCGTCA**T**GCC**G** (R)	24.1	1.3	40.2	0.2	26.1
MLH1	G	TCGTGCTCACGTTCTTCCT**C**C (R)	23.9	1.2	38.5	0.3	14.6
	A	TCGTGCTCACGTTCTTCCA**TT** (R)	24.5	1.2	35.9	0.8	11.4
SUR1	C	TGCCACCCTCCCTCCCTA**C** (F)	23.9	1.1	38.5	0.5	14.6
	T	TGCCACCCTCCCTCCCTA**T** (F)	24.5	1.1	35.9	0.8	11.4
G42863	A	GGCTTGTGGATGAAGGAGA**A** (F)	22.0	1.1	34.9	0.8	12.9
	C	GGCTTGTGGATGAAGGAGT**C** (F)	22.5	1.1	34.8	0.8	12.3
G42888	T	GGGGAGGGGAGGAAGAGAC**T** (F)	21.4	1.1	35.1	0.3	13.7
	C	GGGGAGGGGAGGAAGAGAG**C** (F)	20.9	1.1	35.1	0.7	14.2

[a] Polymorphic nucleotides are given in bold, and introduced mismatched nucleotides to decrease background amplification are underlined; (F) = forward primer, (R) = reverse primer.
[b] Normalized reporter signal minus baseline signal.
[c] C_t of negative allele minus C_t of positive allele

Typing of allelic differences in recipient and donor cells by SNPs

To determine the capacity to discriminate between siblings using real-time PCR for SNPs, 80 SCT recipients and their HLA-identical donors were screened for presence of the seven SNPs. First, the genotype frequency of SNPs in 160 paired siblings was defined (Table 3). This analysis showed that genotype frequency of SNPs in either donors or recipients was similar (data not shown). Moreover, all SNP genotype frequencies that we determined correlated with those published, except one (Table 3). The frequency of the SNP in the *MLH1* gene, we found, differs significantly from that determined in the Japanese population by Ito *et al.*[40]. As expected, frequencies of

heterozygous genotypes for both SNPs located on the X chromosome were low because of single alleles in males (Table 3).

Table 3. Genotype frequencies of SNPs

	PECAM 1			*ICAM1*			*HA1*			*MLH1*			*SUR1*			*G42863*			*G42888*			*n*
	GG	*CG*	*CC*	*GG*	*AA*	*CA*	*CA*	*CATG*	*TG*	*GG*	*GA*	*AA*	*CC*	*CT*	*TT*	*C/CC*	*CA*	*A/AA*	*C/CC*	*CT*	*T/TT*	
Analyzed by PCR	24	46	30	22	42	36	23	42	35	68	25	7	29	48	23	11	34	55	24	47	29	75 F
																24		76	49		51	*85 M*
																29[a]		71[a]	49[a]		51[a]	160[c]
Literature[b]	28	42	30	20	47	33	17	49	34	26	55	19	32	47	21	32[a]		68[a]	40[a]		60[a]	

[a] Allele frequencies.
[b] References are given in Table 1.
[c] Total

Next, we analyzed the contribution of each SNP regarding their capacity to reveal specific molecular markers in sibling-pairs. Exclusive appearance of one of the two polymorphic alleles in either recipient or donor was determined. The seven SNPs revealed a specific marker for either recipient or donor in 24-50% of the pairs (Table 4). The SNP in *MLH1* showed the lowest capacity (24%) to genetically identify cells, because of high frequency of homozygous *MLH1*-G in the analyzed population. SNPs located on the X chromosome contributed as effective in specific recipient or donor marking as SNPs located on autosomes (Table 4). A significant number of sibling pairs (28%) allowed recipient- and donor-specific discrimination by both biallelic variants of one SNP. Hemizygous appearance of allelic SNP-variants on the X chromosome contributed dominantly to this phenomenon (Table 4). Furthermore, the probability of the seven biallelic SNP PCRs to reveal specific markers for recipient and donor was calculated using the genotype frequencies that we had determined in 80 sibling pairs. The analyzed frequencies of SNPs as genomic marker correlated highly with those calculated, suggesting that all SNP-markers segregate in a Medelian fashion that is not influenced by the close relationship of siblings (Table 4).

Table 4. Recipients and donors (%) that can be discriminated based on SNP markers

Number of markers for		PECAM1	ICAM1	HA1	MLH1	SUR1	*G42863*	*G42888*
Recipient or donor[a]	Analyzed by PCR	41	45	30	23	44	35	42
Recipient *and* donor[b]	Analyzed by PCR	3	2	2	1	2	10	8
Total	Analyzed by PCR	44	47	32	24	46	45	50
Total	Calculated[c]	38	35	35	23	39	32	43
Cumulative number of markers for		PECAM1	ICAM1	HA1	MLH1	SUR1	*G42863*	*G42888*
Recipient *or* donor	Analyzed by PCR	44	66	76	83	89	94	97
Recipient *or* donor	Calculated	38	60	74	80	88	92	97
Recipient *and* donor[d]	Analyzed by PCR	3	12	24	32	41	55	67

[a] Either recipient or donor DNA contains exclusively one of the allelic variants of SNPs
[b] Both recipient and donor DNA contain different variants of one SNP
[c] Both recipient and donor DNA contain exclusively one of the allelic variants of SNPs.
[d] Probability that recipient or donor DNA exclusively contain one allele of the SNPs; Calculations were performed using genotype frequencies as analyzed (Table 2).

The high percentages that each SNP could discriminate between siblings resulted in specific markers for either recipient or donor in 97% of these pairs (Table 4). The probability calculations based on genotype frequencies revealed the same discrimination capacity of the seven SNP-markers (Table 4). Each dimorphic SNP increased the percentage of pairs with a specific marker for both recipient and donor significantly (8-14%). Location of two SNPs on the same chromosome (19 and X) did not affect the accumulation of the percentage of sibling pairs with specific SNP-markers (Table 4). The seven SNPs we utilized revealed a specific genomic marker for both recipient and donor in 67% of the 80 analyzed sibling pairs (Table 4). The PCR specific for *SMCY* located on the Y chromosome discriminated recipient and donor cells in al sex mismatched sibling pairs and increased the percentage of pairs that have a specific marker for both recipients and donors with 12% (data not shown).

These results demonstrate that a restricted number of SNPs in the human genome can be used to genotype the vast majority of sibling pairs. Each SNP with a high heterozygous frequency has the ability to discriminate 30-50% of sibling pairs either with a recipient- or donor-specific marker. More importantly, all SNPs used for this analysis act as additional markers specific for either donor or recipient, which results in specific markers for 67% of both members of sibling pairs. Addition of three biallelic SNP-markers that appear to be highly discriminative will lead to specific markers for both recipient and donor in ≥ 99% of sibling pairs.

Quantification of recipient and donor cell ratios

We developed a quantitative assay to measure the percentage of recipient or donor cells by allele-specific real-time PCR in peripheral blood (subsets). Calibration curves for each SNP were performed. Homozygous and heterozygous DNA for all targeted alleles were diluted either in DNA, homozygous for the alternative (negative) alleles, or in water. Amplification-cycle threshold signals (C_t) were plotted against amount of input DNA. Real-time PCR of DNA encoding the albumin gene was simultaneously performed to normalize the amount of input DNA of test samples to the calibration samples. Figure 2 shows calibration curves of amplified SNP-positive DNA (*PECAM1*-GG and *PECAM1*-GC) diluted in negative DNA (*PECAM1*-CC) (Figure 2a) and amplified SNP-positive DNA diluted in water (Figure 2b). All calibration curves of DNA amplified by SNP-specific primer sets reached slopes of -3.3 (± 0.2). Calibration curves for samples diluted in both negative DNA and water gave similar results for all seven SNP-PCR (data not shown). Deviation in C_t was stable and average of two-fold standard error of repeated samples did not exceed 0.4 cycle. The deviation of C_t obtained by real-time PCR is independent of the concentration target DNA in the samples that reached the threshold between 20 and 35 cycles of amplification. Therefore within this range, accuracy was directly related to the concentration and calculated confidential intervals were approximately +30% and -25% for all measured values. As shown in Table 2, all SNP-PCR showed low amplification of DNA containing the allele that was not complementary with the specific SNP-primers (C_t >35). Therefore, specific amplification of 500 ng DNA results in detection up to 0.1% SNP-positive cells in SNP-negative cells. Specific amplification of DNA with SNPs that reached the threshold prior to 22 cycles of amplification ($\Delta C_t > 13$) can be quantified up to 0.01%.

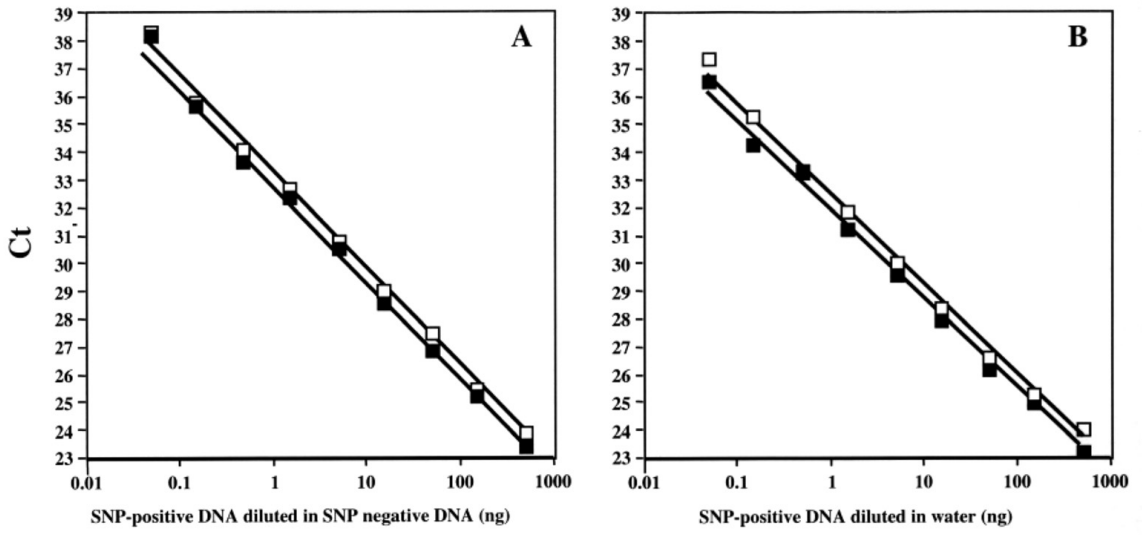

Figure 2. *Calibration curves of DNA. Serial dilutions of DNA containing the G allele of PECAM1 homozygously* (■) *or heterozygously* (□) *(PECAM1-GG and PECAM1-GC, respectively). Standard errors of C_t are ± 0.25 cycle and presented by sizes of the boxes. (a) SNP target sequence-positive DNA diluted in target sequence-negative DNA (PECAM1-CC). (b) SNP target sequence-positive DNA diluted in water.*

The results show that real-time PCR of SNPs can be used to quantify chimerism in cell samples taken after SCT. Quantification of low percentage target-SNP positive cells in mixed samples is very accurate but standard errors are high for samples with high percentage target-SNP positive cells. Analysis of hemopoietic cell populations of patients after SCT by both recipient and donor specific SNP-markers may result in significant information about chimerism present in these patients.

Analysis of recipient-derived hemopoietic cells following SCT and adoptive immunotherapy

To apply our SNP method for the determination of the origin of lymphocytes and myeloid cells after allogeneic transplantation, we studied in detail chimerism of one patient treated for CML, who relapsed and was subsequently treated with DLI (Figure 3). The patient relapsed at 9 months after SCT and received 0.7×10^8 T cells/kg body weight 2 weeks later. Acute GVHD was not observed, but the patient developed extensive chronic GVHD 3 months after DLI. The percentages of recipient-derived T cells and myeloid cells in blood were determined by real-time PCR of the *ICAM1*-G allele. Remarkably, a high percentage of T cells of recipient origin were detected in this patient at 1, 3 and 6 months after SCT. After DLI the percentage of recipient derived T cells decreased rapidly to below the detection limit before the development of chronic GVHD (Figure 3).

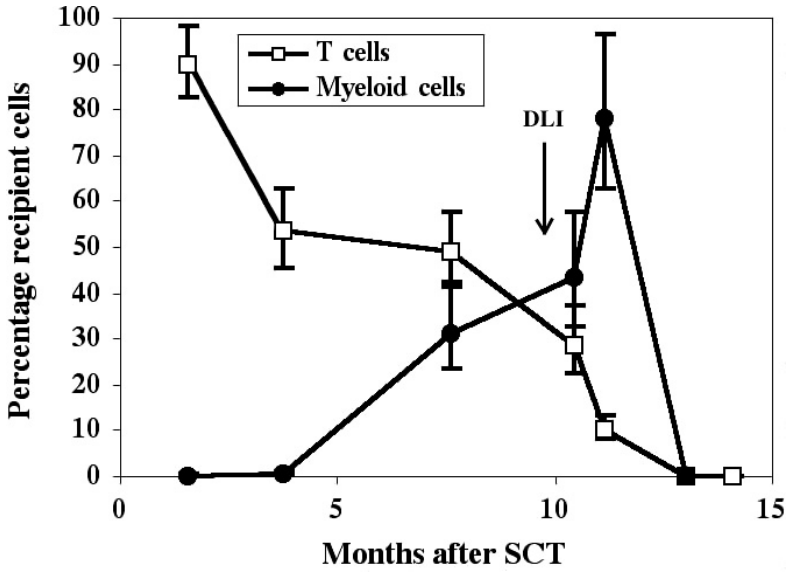

Figure 3. *Analysis of cells of recipient origin after SCT. T cells and myeloid cells are purified by flow cytometry after staining PBMC with cell lineage-specific mAb. The percentage recipient derived T cells (◊) and myeloid cells (■) are detected by real-time PCR with allele-specific primers for polymorphic DNA of the ICAM1 gene. Gray bars represent the confidential interval of real-time PCR of SNP.*

A small percentage of autologous cells could be detected in purified myeloid cells at 1 and 3 months after SCT (0.3 and 0.6%, respectively). However, myeloid cell samples were 99.5% pure, thus contaminating T cells may have contributed to this signal. The percentage of myeloid cells of recipient origin was significantly increased at 7 months after SCT. Shortly after DLI, the percentage of recipient-derived myeloid cells still increased (Figure 3). At two months after DLI the myeloid cells of recipient origin dropped very fast to below 0.05% (Figure 3). A high percentage (50%) Ph+ cells was observed in bone marrow by FISH analysis of at 6 months after SCT. The percentage of Ph+ cells in bone marrow increased up to 90% prior DLI and did not decrease until 1.5 months after DLI. All Ph+ cells disappeared within 5 month after DLI (data not shown).

The data show persistence of high percentage of recipient-derived T cells in this patient after SCT. The percentage of these T cells decreased immediately after DLI before the onset of clinical GVHD symptoms, which suggests that alloreactivity of infused donor cells can be detected early by this method. Moreover, kinetics of myeloid cells of recipient origin in blood after SCT and DLI parallel that of Ph+ cells in BM as analyzed by FISH. This indicates that monitoring of chimerism in myeloid cells may be informative regarding relapse and response to treatment for those myeloid leukemia patients whose leukemia cells do not bear a specific malignancy-marker.

DISCUSSION

The aim of the present study was to develop an accurate, sensitive, and fast method to quantify the percentage of recipient and donor hemopoietic cells after transplantation of HLA-identical stem cells. Following SCT, the majority of transplanted patients have hemopoietic cells of both recipient and donor origin (mixed chimerism) at least for a short period of time. Hemopoietic cells of some of these patients remain of mixed origin for a long period of time or revert to autologous hemopoiesis[46]. The influence of mixed chimerism on leukemic relapse, graft rejection, and treatment failure after SCT has been studied extensively[8,47,48]. It has become clear that the hemopoietic cell lineage in which mixed chimerism occurs contributes to the choice for adoptive immunotherapy after SCT[49,50]. T cell mixed chimerism contributes clearly to graft tolerance and resistance against GVHD[29,30,51]. Mixed myeloid cell chimerism in acute and chronic myeloid leukemia patients may reflect residual disease and may indicate imminent relapse of disease[24,25,27]. Particularly, the kinetics of recipient and donor ratios in these cell lineages could be very informative about immunereactivity and course of disease, and useful in determining strategies of additional treatment[26,27].

We developed a real-time PCR method using seven genomic SNPs as markers that are capable to discriminate either recipient or donor cells in 95% of HLA-identical sibling pairs. Logarithmic diluted concentrations of input target DNA are proportionally linear with C_t. Deviation in C_t is independent of the concentration DNA ranging between 500 ng and 0.05 ng. Therefore, the sensitivity is very high for low concentrations of target DNA but low for high concentrations of target DNA. However, to reach high sensitivity in both directions, two discriminative SNPs can be used with two calibration curves: one calibration curve to quantify a range from 0.1 to

50% recipient cells in donor cells using one SNP-PCR, and one calibration curve to quantify a range from 0.1 to 50% donor cells in recipient cells by another SNP-PCR. One recipient-specific and one donor-specific SNP-PCR can be utilized for sensitive monitoring after SCT and DLI. By this approach seven biallelic SNP-markers resulted in very sensitive monitoring of chimerism in 67% of our patients. Additional real-time PCR analysis with three SNPs allows a screening that can routinely applied to determine specific markers for 99% of both recipients and donors. In addition to SNP-PCR, we developed an SMCY-gene-specific real-time PCR to quantify male cells in sex-mismatched recipient/donor pairs. A similar approach has been described for the DFRY gene by Fehse *et al*.[52]. Male-specific PCR are markers in 50% of in randomly selected recipient and donor pairs and can be utilized as biallelic marker for either recipient or donor. Dimorphic SNPs revealed to be specific markers for either recipient or donor of sibling pairs comparable to male specific markers.

As mentioned earlier, very small percentages of recipient cells in donor cells and *vice versa* can be detected by real-time PCR. A detection limit of 0.01% can be reached using SNP-specific primers that efficiently amplify the positive allele ($C_t \leq 22$) and show low amplification ($C_t > 35$) of the negative alleles. Standard errors in repeated PCR are small (± 0.2 cycle) which results in confidence interval of roughly +30% and -25% of the measured values. However, quantification of SNP-specific DNA by real-time PCR is more accurate than quantification of products by conventional PCRs that amplify DNA samples to a fixed number of cycles. PCR products that differs in length by the number of tandem repeats sequences (STR/VNTR) utilize electrophoresis to quantify both recipient- and donor-specific products amplified in the same PCR. Analysis of recipient cells and donor cells by separate real-time PCR of SNPs excludes interference with nonspecific signals. Moreover, real-time PCR excludes spectral overlap of recipient- and donor-specific fluorescence signals or stutter peaks of amplified recipient DNA, that comigrates with donor specific peaks or *vice versa*, as sometimes observed by amplification of STR[53,54]. In addition, real-time PCR of SNPs is less hampered by competition for reagents that may occur by amplification of PCR products that significantly differ in length. Moreover, the use of reference gene amplified by real-time PCR may better define the amount of input DNA compared to conventional PCR. However, sensitive quantification of recipient- and donor-derived cells in leukocyte subsets may be limited by efficiency of purification.

The percentage recipient and donor pairs that have an SNP-marker is significantly higher than the number of patients and donors that have markers applicable for FISH analysis and can compete with STR markers[55-57]. Use of specific PCR primers for about ten SNPs with high heterozygous frequencies will result in a discriminative marker for both recipient and donor in approximately 99% of all sibling pairs. The reproducibility of our SNP-marker analysis is very high and the method is significantly less laborious compared to marker analysis by FISH. High percentage of recipient and donor cells can be quantified more exactly using FISH analysis[58]. Sensitive detection of low percentages of cells can be done more accurately by real-time PCR than by FISH analysis, because of the restricting number of cells that is used for FISH analysis. Specific target sequences in recipient and donor DNA can be separately utilized for quantification by real-time PCR and thus result in sensitive quantification of both recipient and donor DNA present in one-cell samples.

In summary, monitoring of hemopoietic chimerism after SCT may indicate imminent graft rejection or relapse. Moreover, it gives significant information about the immunological response after SCT and DLI. We show that real-time PCR of SNPs is a sensitive and very reliable method to analyze chimerism. The method is less laborious compared to FISH analysis and quantification is more accurate than that performed after gel electrophoresis and conventional PCR.

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APPENDIX: METHOD IN FOCUS

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Assay characteristics

The quantitatification method is based on a real-time PCR (Perkin-Elmer Applied Biosystems, ABI Prism 7700), with allele-specific primers for DNA sequences containing single nucleotide polymorphisms (SNPs) and target DNA-specific probes[1,2]. The nonextendible hybridization probes are labeled with a reporter fluorescent dye at the 5' end and a quencher at the 3' end, which results in a fluorescence signal after cleavage by 5'-3' nuclease activity of Taq polymerase. A charge-coupled device camera attached to ABI Prism 7700 detects target-specific signals at a threshold of 10 standard deviations above the baseline fluorescence. Normalized signals minus baseline signals of the reporter dyes (ΔR_n) are plotted against PCR cycle numbers, which results in a logarithmic amplification function. The cycle number of DNA amplification that generates the first specific fluorescence signal (ΔR_n) above the threshold is called cycle threshold (C_t). Therefore, C_t represents directly the relative amount of target DNA in the analyzed samples.

Allele-specific amplification revealed that each biallelic SNP acts as a specific marker in 24-50% of 80 sibling pairs. The discriminative capacity of each SNP marker depends on the frequency of the SNPs in the human population. Calculation of the of percentage donor and recipient cells is based on the relation of specific amplification signals from both recipient and donor DNA to specific amplification signals obtained by calibration curves for both recipient and donor markers. The amount of input DNA is simultaneously calibrated by amplifying a DNA fragment of the albumin gene.

Protocol

DNA preparation

Genomic DNA is isolated from PBMC, purified hemopoietic cell populations, and EBV-transformed B cells with QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) following the protocol of the kit. Samples >50 000 cells are eluted with 200 μl and DNA concentrations are determined by spectrophotometry. Samples ≤50 000 cells are eluted with 2 × 25 μl. DNA and the amount of input DNA is only determined by real-time PCR of the albumin gene[3].

DNA from 4 EBV-transformed B cell lines that contain all 14 SNP alleles are used as calibrators. Aliquots (20 μl) of DNA dilutions, containing 100, 30, 10, 3, 1, 0.3, 0.1 and 0.03 ng/μl, are stored at –80 ºC. In total, 5 μl of these dilutions are amplified by allele-specific real-time SNP-PCR and by the albumin gene-specific real-time PCR to construct calibration curves.

PCR

Taqman-based real-time PCR for each of the SNPs, SMCY and albumin has been performed in a total volume of 50 μl with 1 × PE sample buffer, 1.25 U of DNA polymerase (AmpliTaq Gold, Perkin-Elmer), 250 μM dNTP, primers at 300 nM, and

TET-labeled probes at 100 nM. Hot start Taq polymerase is activated for 10 min at 95°C. DNA is amplified for 45 cycles. Screening of recipient and donor DNA with all SNP-specific PCR occurs in one run at annealing/extension temperature of 60°C. Allele-specific primers for *PECAM1* and G42863; G42888 are used for this application (Table 1). Annealing and extension of quantitative PCR (calibration curves and test samples) takes place for 60 seconds at a temperature optimum dependent on the primer sets used (Table 1). Annealing and extension is followed by denaturation for 15 s at 95°C.

Analysis of PCR products

Amplification curves are constructed by plotting PCR cycle number versus target-specific fluorescence signals of the reporter dye (ΔR_n). The cycle number that generates the first fluorescence signal (ΔR_n) above the threshold is called cycle threshold (C_t) and represents the relative amount of input target DNA.

Quantification of donor chimerism

Quantitative analysis is performed by generating calibration functions from C_t obtained by real-time PCR of DNA serially diluted in water (500-0.05 ng) isolated from cell samples bearing SNPs. Simultaneously, allele-specific target DNA (SNP) and DNA encoding the non-polymorphic albumin sequence are amplified. The calibration functions are

$$y = a^{10} \log x + b$$

in which a is the slope of the curve and b is the intercept with the y-axis.

The percentage of recipient or donor target cells, is calculated by the following formula:

$$\text{SNP positive DNA (\%)} = 10^{(y_1-b_1)/a_1-(y_2-b_2)/a_2} \times 100\%$$

where

y_1 is the C_t sample, amplified by allele-specific SNP-PCR,

y_2 is the C_t sample, amplified by albumin PCR, b_1 is the intercept with the y-axis of allele-specific SNP-PCR calibration curve,

b_2 is the intercept with the y-axis of albumin PCR calibration curve, a_1 is the slope of the curve of allele-specific SNP-PCR calibration curve, and

a_2 is the slope of the curve of albumin PCR calibration curve.

The slope of all calibration curves is -3.3 (±0.2). The detection limit for each individual sample is dependent on the C_t of albumin PCR (y_2) and the intercept with y-axis of allele-specific SNP-PCR (b_1) and the amount of template DNA: the intercept with y-axis of albumin PCR (b_2). Table 1 shows the detection limits for al SNP-PCR using a cut-off level at C_t >35 cycles.

Time requirement

Screening test to discriminate recipient from donor DNA

Total time: 3.5 h; hands-on time: 1.5 h. The time indicated represents total time required for DNA isolation and PCR preparation, assuming 30 min hands-on time for DNA isolation of cell samples of one recipient/donor pair. We use ready-to-use PCR master mix in optical tubes that are stored at -20°C for all allele-specific SNP-PCR. Preparing master mix for 8 recipient/donor pairs takes about 3 h. One real-time PCR run (45 cycles) takes 2 h. Analysis of the data of the screenings assay of 8 recipient/donor pair takes 1 h. Combining a number of recipient/donor pair in one assay reduces the time considerably.

Quantitative chimerism analysis of sorted cell samples

Cell sorting
Total time: 2 h per sample (two subpopulations). T cells and myeloid cells are isolated by flow cytometry (Epics Elite, Beckman Coulter, Fullerton, CA, USA) from liquid nitrogen stored PBMC. Both T cells and myeloid cells are sorted from one sample.

DNA-handling and real-time PCR
Total time: 5 h; hands-on time: 3 h. Again, 30 min hands-on time for isolating DNA of each sample (both lymphoid and myeloid) is assumed. We use ready-to-use calibration curve samples that are stored at -20°C for all allele-specific SNP-PCR. About 1.5 h is required to prepare the DNA samples for quantitative PCR, in general 28 tubes (eight tubes SNP-PCR calibration curve, eight tubes albumin-PCR calibration curve, six tubes per purified cell sample; two tubes positive SNP-PCR, two tubes negative SNP-PCR and two tubes albumin PCR). One real-time PCR run (45 cycles) takes 2 h. In addition, 1h is required to analyze the results.

Sensitivity

We include a cut-off level at C_t 35 since we observed that calibration curves are not always linear when very low amounts of target DNA are amplified. This results in a sensitivity of ≥0.01% when the signal of SNP-positive DNA (100%) reaches the threshold ≤23 cycles and background amplification of the negative allele reaches threshold after 35 cycles (ΔCt = 12). This is reached by an input of about 500 ng DNA. Roughly, ΔCt of three cycles represents a 10-fold quantitative difference, fulfilling the condition that the slope of the calibration function closely fits -3.3. Therefore, using smaller amounts of input DNA, for example 50 ng, will decrease the sensitivity to ≥0.1%. In contrast, higher amounts of input DNA will result in higher sensitivity for all SNP-PCR that show low background amplification.

Table 1. Specific characteristics of real-time PCR of SNPs

Gene/STR	SNP	Allele-specific primer (5'-3')[a]	Common primer (5'-3')	Target site probe[b] (bp)	PCR temp.	Detection limit (%) input DNA	
						500	50 ng
PECAM1	C G	(AG)GACTCACCTTCCACCAACCCG^{R} (A)GGACTCACCTTCCACCAACCTC^{R}	GGATCTATGACTCAGGGACATATAAATG^{F}	37-68	63	0.01 0.06	0.1 0.6
ICAM1	G A	AGAGCACATTCACGGTCACCCAC^{R} AGAGCACATTCACGGTCACCATT^{R}	GCACTTTCCCACTGCCCAT^{F}	28-56	60	0.09 0.06	0.9 0.6
HA1	C-A T-G	GCTCTCACCGTCACGCAA^{R} GCTCTCACCGTCATGCCG^{R}	TGCTGGCGGACGTGG^{F}	17-36	62	0.07 0.04	0.7 0.4
MLH!	G A	TCGTGCTCACGTTCTTCCTCC^{R} TCGTGCTCACGTTCTTCCATT^{R}	GAGACCCAGCAACCCACAGA^{F}	40-67	62	0.03 0.05	0.3 0.5
SUR1	C T	TGCCACCCTCCCTCCCTAC^{F} TGCCACCCTCCCTCCCTAT^{F}	GTCTTCCAGAGTCCCCGACAG^{R}	45-65	64	0.03 0.05	0.3 0.5
G42863	A C	(G)GCTTGTGGATGAAGGAGAA^{F} GGCTTGTGGATGAAGGAGTC^{F}	TGGCACATCTGGCAAAATCTC^{R}	23-52	65	0.01 0.01	<0.1 <0.1
G42888	T C	(G)GGGAGGGGAGGAAGAGACT^{F} (G)GGGAGGGGAGGAAGAGAGC^{F}	TTGGTGCTGCTGTAATCACTTTTCAT^{R}	54-83	62	0.01 0.01	<0.1 <0.3
SMCY		TCTTGCGTCCTCA^{F}	AGTGTGGTACGAGCCGTCTCA^{R}	22-48	62	0.03	0.3

[a]Nucleotides (in parentheses) are removed for annealing at 60°C used for SNPscreening; ^{F} = forward primer, ^{R} = reverse primer.

[b]Related to the first nucleotide of the forward primer.

Specificity/informativeness

Aspecific amplification with the developed real-time PCR to specific amplify SNP is not observed. Amplification of DNA containing the negative SNP-alleles is below the cut-off level (Ct > 35 cycles).

The capacity of biallelic polymorphic markers to discriminate siblings is calculated from published SNP frequencies. We selected SNP markers that allowed discrimination between recipient and donor in >30% of sibling pairs. The DNA of 80 sibling pairs is analyzed for all 7 SNP markers by real-time PCR. The SNP marker in G42888 is most efficient and identifies recipient or donor cells in 50% of sibling pairs. SNP markers in *ICAM1, SUR1*, *PECAM1,* G42863, and *HA1* identify donor or recipient cells in 47, 44, 46, 45 and 32% of sibling pairs, respectively. However, the SNP marker in *MLH1* is significantly less efficient than calculated from published genotype frequencies (24 *vs* 44%) due to discrepancy in genotype frequencies in the analyzed populations. Combining all seven biallelic SNPs results in genotype-specific markers for recipient or donor in 97% of sibling pairs. Two-way discrimination (recipient and donor) is feasible in 67% of sibling pairs by use of two SNP markers. The determination of both the percentage recipient and donor cells improves significantly the sensitivity and accuracy of the method.

Reproducibility/accuracy

Reproducibility is high; the variability of independent analyses in general does not exceed the deviation of these samples. To exclude trivial errors, all sample analyses are performed in duplicated wells. Standard errors of calibration curves (Δy) obtained from C_t of at least eight dilutions do not exceed 0.2 cycle. The deviation of C_t obtained by real-time PCR is independent of the concentration target DNA at least for $20 \leq C_t \leq 35$. Therefore, accuracy (Δx) is directly related to the concentration and can be calculated by the regression curve ($y = a^{10}\log x + b$) by the formula: $\Delta x/x = +10^{\Delta y/a}-1$ and $-10^{\Delta y/a}-1$. DNA amplification by the developed SNP-PCR are stable logarithmic processes and slopes (a) of calibration curves are -3.3 (±0.2).

Using a maximal Δy (Δy calibration curve + Δy sample = 0.4) and slopes of -3.1, the maximal deviation can be calculated and reveals that $\Delta x = -0.26x$ and $+0.30x$. Table 2 shows the calculated confidential intervals for percentages between 100 and 0.1.

In summary, due to the logarithmic amplification by PCR, small percentages of target DNA can be accurately defined. Absolute confidential intervals between 0.25% (-0.065 and +0.075) and 0.5% (-0.13 and +0.15) will be statistically significant. However, high percentages of target DNA have high absolute confidential intervals (for example, measured value of 25% is at least between 18.5% and 32.5% target DNA, and a measured value of 75% is at least between 55.5% and 100% target DNA).

Cost of the assay

The overall cost per analysis is dependent on the total number of analysis performed. The numbers indicated are based on one blood sample, of which both myeloid and lymphoid cell are analyzed. Normally, more samples are combined in one assay, which reduces assay cost considerably.

(1) DNA isolation (Qiagen): 3 euros per sample
(2) Taqman consumables: 17 euros per sample

Trouble shooting

Real-time PCR by allele-specific primers is extremely reproducible and is only affected by inhibitory factors in DNA. Most problems are related to the use of the ABI Prism 7700 PCR technique. For detailed information about troubleshooting see user's manual 'ABI Prism 7700 Sequence Detection System', (Perkin-Elmer Applied Biosystems).

(1) Low signal after amplification of reference DNA
DNA concentration too low — *Increase the amount of input DNA*
(2) Slope calibration curves not between -3.1 and -3.5 or standard error >0.2 cycles
Bad quality of input DNA — *Use another DNA batch for the generation of calibration curves*
(3) Differences between C_t of two-fold repeated samples >0.2 cycles
Always due to trivial errors (polluted tube, no DNA input in one tube, etc) — *Repeat PCR*
(4) C_t of negative DNA (DNA that only contains the negative allele) < 33
Input of too high a concentration of DNA as shown by low C_t of albumin PCR — *Decrease the amount of input DNA*
PCR conditions not optimal as shown by normal C_t of albumin PCR — *Change PCR conditions*

Materials

Hardware

(1) Waterbath. (2) Microtube centrifuge. (3) Spectrophotometer. (4) PE Applied Biosystems PRISM 7700.

Reagents and solutions

(1) Genomic DNA. (2) Taqman kit 1000 reactions with buffer A. (3) Primers (Eurogentec, Seraing, Belgium). (4) Probes (PE Applied Biosystems, Cheshire, UK).

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CHAPTER 6

Intensification of the conditioning regimen with idarubicine before partially T cell-depleted allogeneic stem cell transplantation for chronic myeloid leukemia enhances the conversion of T cells to donor origin and improves clinical outcome

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ABSTRACT

We studied the clinical implications of intensification of the conditioning regimen in patients with chronic myeloid leukemia in first chronic phase (CML-CP1) transplanted with partially T cell-depleted stem cell grafts from HLA-identical siblings. In addition, we studied the influence of intensification of the conditioning regimen on the kinetics of hematopoietic conversion from patient to donor in lymphoid and myeloid subsets. Nineteen patients received conditioning with cyclophosphamide and total body irradiation (TBI), and 70 patients received a conditioning regimen intensified by the addition of idarubicine.

Addition of the chemotherapeutic drug idarubicine resulted in a significant reduction of the 5-year probability of relapse from 69 to 40% ($p = 0.002$). The 5-year probability of leukemia-free survival (LFS) was significantly higher for patients who received the intensified conditioning. However, the 5-year probability of current LFS, defined as survival in first or second remission, was identical for both conditioning regimens. This was mainly caused by a favourable response of patients with CML to therapeutic donor leukocyte infusion (DLI). The addition of idarubicine correlated with an increase in the incidence and severity of acute graft-versus-host disease (GVHD), albeit clinically mild. Addition of idarubicine did not influence transplant-related mortality.

We monitored hematopoietic chimerism after stem cell transplantation (SCT) in lymphoid and myeloid subsets using a recently developed real-time quantitative PCR for the detection of recipient/donor-specific single nucleotide polymorphisms. The increase in acute GVHD and LFS after intensification of the conditioning regimen with idarubicine coincided with a faster increase in chimerism in both $CD4^+$ and $CD8^+$ T cell subsets. The addition of idarubicine to the conditioning had no differential effect on the time to reach full chimerism in non-T cell subsets. These chimerism dynamics may confirm the importance of the genotype of immune mediators in the development of allogeneic responses. Furthermore, they imply the importance of T cells in the development of allogeneic reactivity. This observation may have consequences for the conditioning regimens before T cell-depleted SCT of patients with hematological malignancies that do not respond well to therapeutic DLI.

INTRODUCTION

ALLOGENEIC STEM CELL TRANSPLANTATION (SCT) is the only curative treatment option for patients with chronic myeloid leukemia (CML)[1,2]. Transplantation of stem cells from a donor results in the effective replacement of recipient stem cells that are eradicated after total body irradiation (TBI) and chemotherapy. Immunoreactivity of donor-derived T cells against residual malignant cells, referred to as graft-versus-

leukemia (GVL) reactivity, contributes to the success of this form of therapy. However, donor T cells may recognize minor histocompatibility antigens that are not exclusively expressed by the leukemic clone, resulting in immunoreactivity towards normal host tissues. This broad alloreactivity can result in the development of graft-versus-host disease (GVHD). GVHD remains one of the major causes of morbidity and mortality after non-T cell-depleted SCT. Moderate to severe acute GVHD (grade 2-4) occurs in about 45% of recipients of HLA-identical marrow grafts and contributes to death in 20-70% of those affected[3-5]. Approximately 30-50% of the patients develop chronic GVHD which is also associated with significant morbidity and mortality (50%) as a result of long term immunosuppression and consequently severe immune dysfunction[6]. One of the most effective methods to reduce GVHD after allogeneic SCT is depletion of T cells from the donor graft. However, the removal of immunocompetent T lymphocytes is associated with an increase of graft rejection and relapse of leukemia[2,7-13]. A strategy to prevent relapse after T cell-depleted SCT is the intensification of the conditioning regimen. We demonstrated a favourable outcome after intensification of the conditioning regimen with the addition of idarubicine in standard risk leukemia patients[14]. Furthermore, we hypothesized that the addition of idarubicine may exert an immunomodulatory effect[14].

In this study we present the outcome of allogeneic SCT with or without an intensified conditioning regimen in a large cohort of 89 patients transplanted for chronic myeloid leukemia in first chronic phase (CML-CP1). Moreover, chimerism was monitored sequentially in highly purified subsets of peripheral blood mononuclear cells (PBMCs) and granulocytes of 10 patients randomized between recipients of allogeneic SCT with or without intensification of the conditioning regimen, using a newly developed real-time PCR for the quantification of hematopoietic chimerism[15,16]. The results reaffirm that the addition of the chemotherapeutic drug idarubicine results in a favourable outcome, demonstrated by a significant reduction of the 5-year probability of relapse, and a significantly higher 5-year probability of leukemia-free survival (LFS). Increase in incidence and severity of acute GVHD is clinically mild, and did not influence transplant-related mortality.

Several mechanisms may contribute to the increased occurrence of GVHD and probability of LFS. Intensification of the conditioning regimen may cause more extensive injury to the mucosal barrier resulting in higher levels of pro-inflammatory cytokines, and subsequent increase in GVH reactivity[17,18]. The addition of idarubicine may directly increase the cytoreduction of leukemic cells. Finally, idarubicine-mediated cytoreduction of autologous T cells may enhance the engraftment of donor T cells, and therefore indirectly result in an increase of GVH reactivity.

In this study we demonstrated that intensification of the conditioning regimen with idarubicine results in a faster increase in chimerism in T cell subsets. Several studies have demonstrated the correlation of a reduced incidence of GVHD and increased occurrence of relapse with mixed chimerism[19-21]. The ability to enhance conversion to full hematopoietic chimerism with a cytoreductive drug may have important consequences especially for patients with hematological malignancies that do not respond well to immune mediated cellular therapy, such as SCT and (pre-emptive) donor leukocyte infusion (DLI).

PATIENTS AND METHODS

Patients and donors

Between March 1984 and December 2000, eighty-nine consecutive patients received allogeneic stem cell transplantation for CML-CP1. Donors were HLA-A, -B, -DRB, -DQB identical siblings. The median age of the recipients (48 males and 41 females) and donors (54 males and 35 females) was 40 years (range 16-61 and 13-71, respectively) (Table 1). All recipients and donors or their guardians gave their informed consent. The ethics committee of the University Medical Center St Radboud, Nijmegen, approved all protocols and consent forms.

Chemotherapy before SCT

Before SCT all 89 patients were treated with hydroxyurea. Nine patients received additional interferon alpha (Table 1). Interferon was stopped three months before SCT.

Conditioning regimen

Nineteen patients received conditioning consisting of cyclophosphamide (Cy) administered intravenously (60 mg/kg body weight) on each of two consecutive days (day –6 and –5), followed by fractionated total body irradiation (TBI) in two equal fractions on day –2 and –1 to a total dose of 9 Gy or 12 Gy. Seventy patients received an intensification of the conditioning regimen with the intravenous infusion of demethoxy-daunorubicine (idarubicine) to a total dose of 42 mg/m^2 body surface on days –12 and –11, followed by Cy (60 mg/kg body weight) on days –6 and –5 and fractionated TBI to a total dose of 9 Gy on day –2 and –1. Donor marrow was partially depleted of T lymphocytes by density gradient centrifugation followed by counterflow centrifugation as previously described[22,23] and infused 24 hours after completion of TBI. The median number of T cells in the marrow grafts was 0.7 (range 0.4-1.9) × 10^6 CD3$^+$ cells/kg body weight. Patients received prophylactic antibiotics according to institutional guidelines. All patients were managed in single rooms with filtered air under positive pressure during their hospital stay.

GVHD prophylaxis

Immunoprophylaxis after transplantation consisted of cyclosporine A (CsA) 3 mg/kg/day by continuous intravenous administration from day -1 to +14. Thereafter CsA dose was reduced to 2 mg/kg/day and continued until day 21. Beyond day 21, CsA was administered orally at a dose of 6 mg/kg/day until 8 to 10 weeks after SCT. In the absence of GVHD CsA was gradually tapered and eventually discontinued at 12 weeks post grafting.

Table 1. Patient and donor characteristics and treatment-related variables.

	Conditioned with idarubicine	*Conditioned without idarubicine*
N° of patients evaluated	70	19
N° of male/female recipients	36/34	12/7
N° of male/female donors	41/29	13/6
Donor/recipient gender (N°)		
male to male	17	10
male to female	16	2
female to female	12	4
female to male	25	3
Median age patients (years)	40	40
Median age donors	42	37
N° pre-treated with IFN-α	6	3
Conditioning regimen		
Cy (60 mg/kg)	70	19
TBI (9 Gy)	70	13
TBI (12Gy)	0	6
N° of T cells in the graft	0.7×10^6/kg	0.7×10^6/kg
Follow-up	July 1986-January 2002	March 1984-January 2002

IFN-α, interferon-α; Cy, cyclophosphamide; TBI, total body irradiation.

Assessment of hematopoietic chimerism

Hematopoietic chimerism was determined using cytogenetic analysis, red blood cell phenotyping (RCP), and real-time PCR.

Cytogenetic analysis was performed on unstimulated bone marrow cells (myelo-monocytic and erythrocytic progenitor cells) and PHA-stimulated T cells of all 89 recipients. Additional differentiation was performed by fluorescent *in situ* hybridization: (FISH) on heterosome interphases (400) of sex-mismatched recipient-donor couples. In patients with a discriminating marker (n = 72) analysis was performed routinely before SCT, 6 and 12 months after BMT and annually thereafter as described previously[24]. The median number of bone marrow metaphases analysed was 31 (range 14-34) and the median number of metaphases of lymphocytes was 32 (range 8-32), allowing a sensitivity level of 10-12%[25].
RCP was performed using a flow cytometric and/or fluorescent microscopic microsphere method as described previously[26-28]. All patients had a discriminating patient and/or donor marker. Patients were evaluated 1, 2, 3, 6, and 12 months after SCT and annually thereafter.

Real-time PCR for single nucleotide polymorphisms (SNPs) and/or the SMCY gene[15,16] was performed on granulocytes and purified peripheral blood mononuclear

cell (PBMC) subsets of 10 patients that were randomized between the groups that received the intensified or non-intensified conditioning regimen. Chimerism was analyzed 1, 3, 6, 12 months after SCT and annually thereafter.

Isolation of PBMC subsets and granulocytes

Granulocytes were separated from mononuclear cells by Ficoll-Hypaque density gradient centrifugation. Mononuclear cells were purified using a magnetic cell separation technique (MiniMacs, Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Mononuclear subsets were defined as follows: $CD3^+/CD4^+$ (Th lymphocytes), $CD3^+/CD8^+$ (Tc lymphocytes), $CD45^+/CD19^+$ (B lymphocytes), $CD45^+/CD14^+$ (monocytes), $CD45^+/CD15^+$ (granulocytes), and $CD3^-/CD56^+$ (NK cells). After direct labelling with anti-CD4, -CD8, -CD19 and -CD14 Microbeads (Miltenyi Biotec), subsets were magnetically isolated on MiniMacs columns, to a purity of 93-97%. Magnetic sorted subsets were re-incubated with fluorescent antibodies specific for each cell population and resorted on the flow cytometer to obtain a purity of more than 98%. Granulocytes were isolated after lysis of the red blood cells and subsequent incubation with anti-CD45-FITC and anti-CD15-PE, using flow cytometric sorting (Coulter-Elite, Miami Fl, USA). CD56-PE-positive NK cells were sorted excluding CD3-FITC-positive staining cells.

Definitions

The first day of engraftment was defined as the first of 3 consecutive days with peripheral white blood cell (WBC) counts $\geq 10^9$/l. Graft failure was defined as primary when the WBC count remained $< 10^9$/l, and secondary when WBC count was $\geq 10^9$/l on 3 or more consecutive days and subsequently dropped to $< 10^9$/l during follow-up.

Relapse in this study was defined as the occurrence of either, or the combination of, hematological, cytogenetic, and molecular relapse. Hematological relapse was defined as the reappearance of clinical features and laboratory characteristics of CML. Cytogenetic relapse was defined as recurrence of metaphases of interphases containing the Philadelphia chromosome. Molecular relapse was defined as a positive PCR at two or more consecutive points with a tenfold increase of the Bcr-Abl fusion transcript measured by real-time RT-PCR[29].

Survival was defined as alive at the end of follow-up. Leukemia-free survival was defined as survival in first hematological, cytogenetic, and, if available, molecular remission. Current leukemia-free survival was defined as remission in first or second hematological, cytogenetic and molecular remission, and remaining in that remission until the end of follow-up. For these patients the follow-up was also defined as from SCT onwards.

Follow-up

Follow-up was until January 1st 2002 or prior death. Survival and relapse were followed to the time of last contact with the patient. The median survival of the patients alive at end of follow-up (n = 58) was 100 months (range 10-187).

Statistics

Fisher's exact test and Chi square test were used for comparison of two proportions. The probability of relapse, leukemia-free survival, current leukemia-free survival and survival were calculated using the Kaplan-Meier product limit method. Differences in probabilities were calculated using the Wilcoxon's log rank test. Differences in percentages of the sequentially evaluated autologous $CD4^+$ and $CD8^+$ cells between the patients conditioned with and without intensification with idarubicine were tested using ANOVA univariate analysis.

RESULTS

Engraftment

Graft failure occurred in 3/89 patients. One patient showed no signs of engraftment (primary graft failure), while 2 patients rejected their grafts (secondary graft failure). All of these patients received idarubicine in the conditioning regimen. Patients were retransplanted with an unmanipulated graft after fractionated total lymphoid irradiation (total 12 Gy). One patient died of fungal infection before he was evaluable for engraftment. Another patient died of severe grade 4 acute GVHD 3 months after retransplantation. One patient remained in molecular remission 110 months after the first SCT and 108 months after retransplantation.

Development of GVHD

The incidence and severity of acute and chronic GVHD after allogeneic SCT are summarized in Table 2. Ninety-three percent (83/89) of patients were evaluable for acute GVHD. The incidence of acute GVHD was significantly higher in patients conditioned with idarubicine than in patients that received no intensification of the conditioning regimen (70 and 37%, respectively; $p = 0.015$, chi square). Severity of acute GVHD, however, was clinically mild (grade 1 and 2). Severe acute GVHD (grade 3 and 4) occurred in only 1 out of 67 patients conditioned with the intensified regimen and in 1 out of 16 patients who were given no intensification of the conditioning regimen ($p > 0.05$).

There was a trend for a higher incidence of chronic GVHD after intensification of the conditioning regimen (48 over 33%; $p = 0.3$). Extensive chronic GVHD occurred more frequently after intensification of the conditioning regimen ($p = 0.16$). However, the incidence of extensive chronic GVHD was low in both groups (12% and 0%, respectively). Twenty-eight patients received therapeutic DLI after relapse. After DLI 8/28 patients developed ≥ grade 2 acute GVHD, 6/28 developed chronic limited GVHD and 2/28 patients developed extensive chronic GVHD.

Table 2. Incidence of GVHD[a] in evaluable patients conditioned with or without the addition of idarubicin.

	Percentage of patients (n)	
	Conditioned with idarubicine (n = 67)	Conditioned without idarubicine (n = 19)
Acute GVHD		
Grade 1	49 (33)	31 (5)
Grade 2	20 (13)	0 (0)
Grade 3	0 (0)	0 (0)
Grade 4	1 (1)	6 (1)
Chronic GVHD		
Limited	36 (21)	33 (5)
Extensive	12 (7)	0 (0)

[a]The clinical manifestations of acute GVHD were classified from grade 1 to 4, according to the criteria described by Glucksberg, *et al*[46]. Chronic GVHD was graded as limited or extensive as described by Shulman *et al*[47].

Mortality after allogeneic SCT

Thirty-one of 89 patients (35%) died after SCT. Principal causes of death were relapse (n = 10), GVHD-related causes (n = 6), infection (n = 6), acute respiratory distress syndrome (n = 2), veno-occlusive disease (n = 2), secondary malignancy (n = 2), failure of engraftment (n =1), miscellaneous (n = 2). Nine out of 19 patients (47%) conditioned without idarubicine died. Overall mortality in the patients conditioned with idarubicine was 22 out of 70 (31%). Non-relapse mortality was 32% in the patients conditioned without idarubicine and 21% in the patients conditioned with idarubicine. The day-100 mortality after SCT was lower in the patients conditioned with idarubicine although this difference was not significant (21% versus 6%, p = 0.06).

Intensification of the conditioning regimen with idarubicine reduces the probability of relapse of CML-CP1 after allogeneic SCT

Figure 1a shows the probability of relapse in patients after allogeneic SCT with and without intensification of the conditioning regimen. Patients conditioned with the intensified conditioning regimen had a significantly lower probability of relapse (p = 0.002) compared with the patients conditioned without idarubicin.

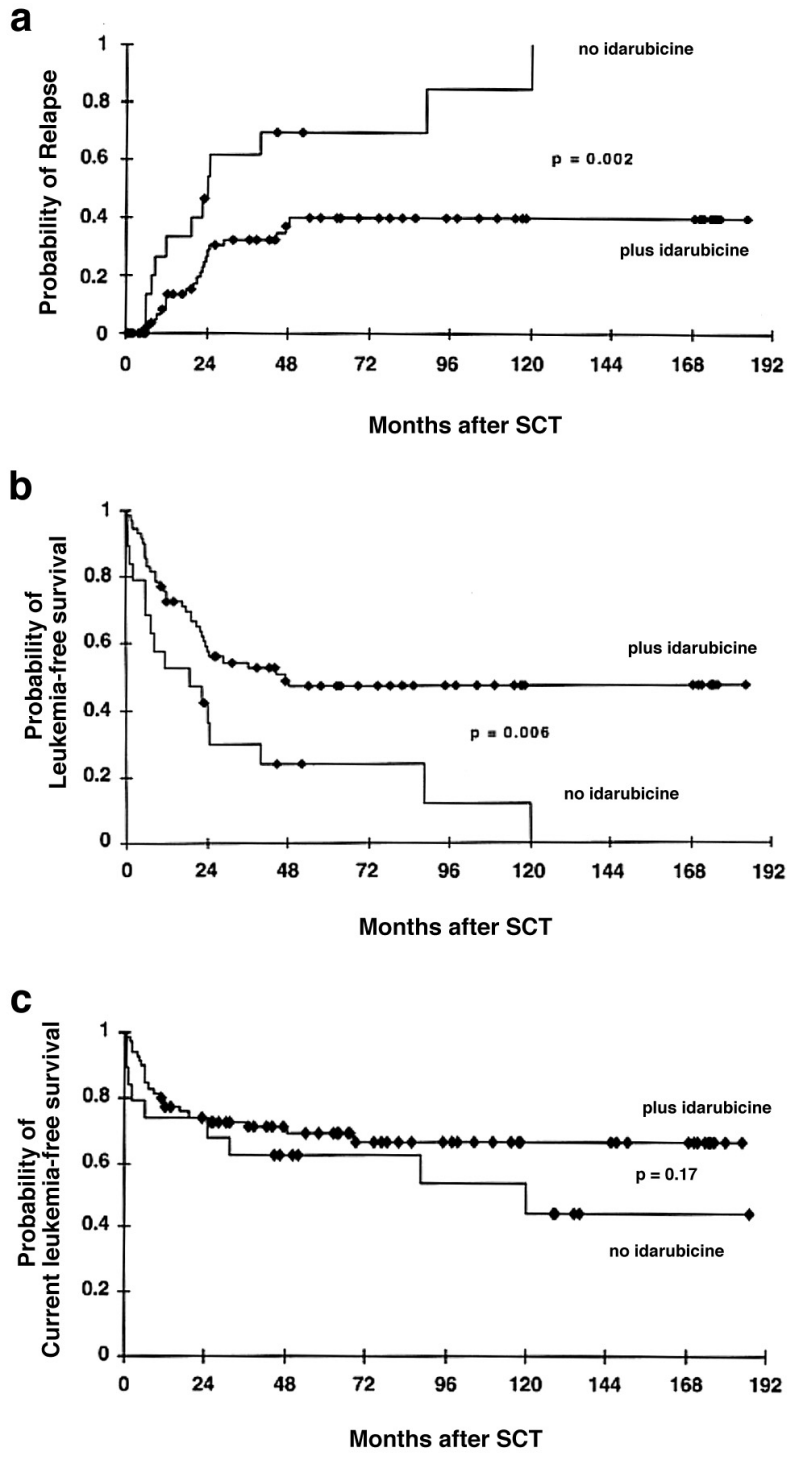

Figure 1. *Probability of relapse (a), leukemia-free-survival (b), and current leukemia-free-survival (c), after SCT for CML-CP1 patients conditioned with or without intensification of the conditioning regimen with idarubicine*

The 5-year probability of relapse for the patients conditioned with and without idarubicine was 40 and 69%, respectively (Table 3).

Table 3. Five-year probability of relapse, leukemia-free survival, survival and current leukemia-free survival and P values for patients conditioned with and without the addition of idarubicine.

5-year probability of	Percentage (95% Confidence Interval)		*p*-value[a]
	With idarubicine	***Without idarubicine***	
Relapse	40 (26-54)	69 (45-93)	$p = 0.002$*
Survival	72 (61-83)	68 (47-89)	$p = 0.14$
LFS	47 (31-63)	24 (4-44)	$p = 0.006$*
Current LFS	69 (57-81)	62 (41-83)	$p = 0.17$

[a]*p*-value of differences in treatment outcome of patients conditioned with idarubicine versus no idarubicine, using two-tailed Log Rank test. *P < 0.05.

Intensification of the conditioning regimen with idarubicine increases the probability of leukemia-free survival after allogeneic SCT

The 5-year probability of survival for the patients conditioned with the intensified regimen was not different from the 5-year probability of survival in the patients conditioned without idarubicine (72 and 68%, respectively; p = 0.14) (Table 3). However, 5-year probability of leukemia-free survival (LFS) for the patients conditioned with the intensified conditioning was significantly higher compared with the probability of LFS obtained in the patients conditioned without idarubicine (47 and 24%, respectively; p = 0.006) (Table 3). The probabilities of LFS for the two different conditioning regimens are depicted in figure 1b.

Patients who remained in complete remission or who responded to therapeutic donor leukocyte infusions (DLI)[30] with a second complete hematological, cytogenetic and molecular remission, were considered 'current' leukemia-free survivors. Twenty-eight patients received DLI with a median number of 0.7 (range 0.1–2.8) × 10^8 $CD3^+$ lymphocytes/kg body weight. Nine were from the group without idarubicine (*i.e.* 47% of this group) and 19 patients received the intensified conditioning (*i.e.* 27% of this group). Both groups responded equally well (70 (7/9) and 72% (13/19), respectively) to DLI, causing the current LFS to be equal (p = 0.17).

Analysis of hematopoietic chimerism using RCP provides a good marker for relapse

Hematopoietic chimerism was assessed at 6 and 12 months after SCT using red blood cell phenotyping (RCP) and/or cytogenetic analysis. There was a good correlation between the increasing number of autologous red blood cells (as part of the CML-clone), the reappearance of Philadelphia chromosome positive metaphases, FISH-positivity for the Philadelphia translocation, and the quantitative amount of Bcr-Abl fusion transcripts measured by quantitative real-time RT-PCR (data not shown). Figure 2a shows the correlation of different relapse markers with autologous red blood cell hematopoiesis as determined by RCP for UPN 480.

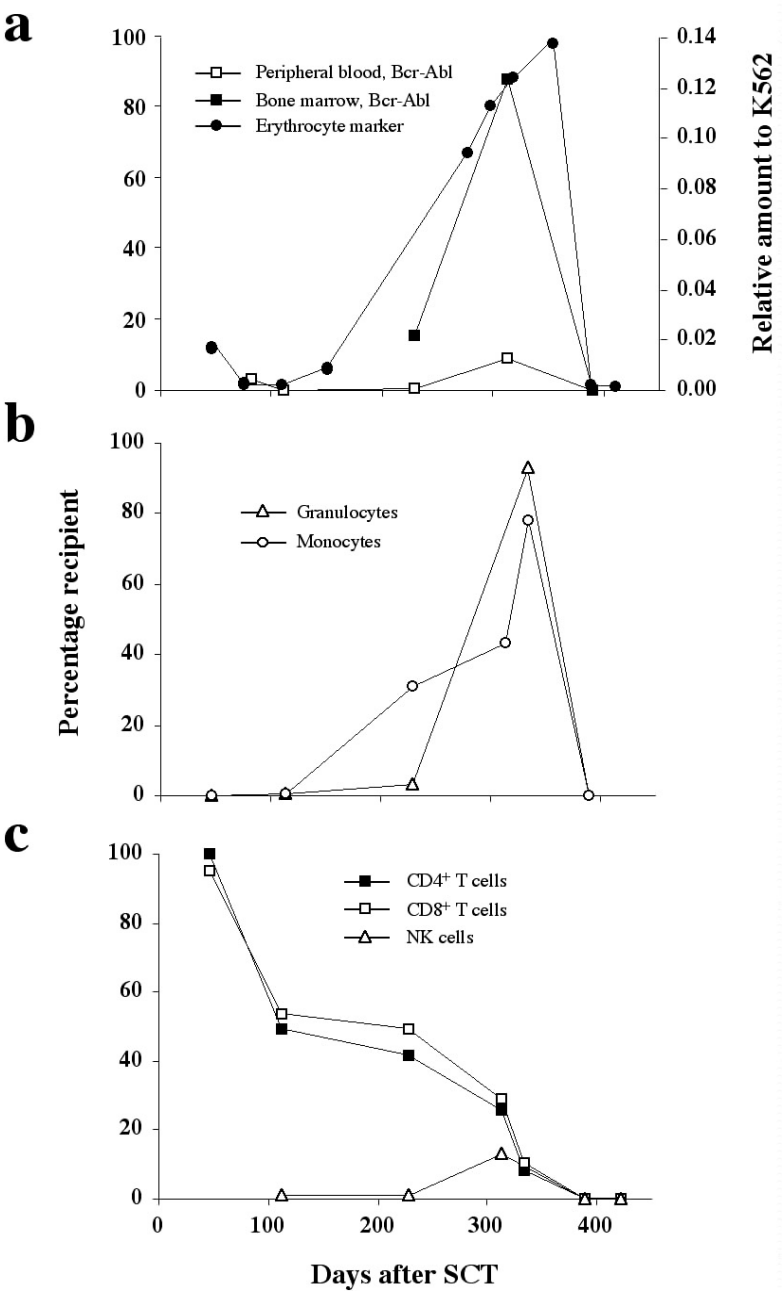

Figure 2. *Percentage of autologous cells in hematopoietic subsets of UPN 480 after allogeneic SCT. (a) Percentage recipient red blood cells combined with the amount of the Bcr-Abl fusion transcript real-time quantitative RT-PCR in blood and bone marrow, relative to expression in K562 cell line. (b) Percentage of recipient granulocytes and monocytes in the peripheral blood. (c) Percentage of recipient peripheral blood NK and CD4+ and CD8+ T cells. DLI was given at day 292 after SCT.*

Using RCP and cytogenetics no difference was observed in the number of patients with mixed chimerism after SCT who were conditioned with or without idarubicine (data not shown).

Quantitative analysis of chimerism in purified subsets of PBMC and granulocytes

Several studies have demonstrated the role of hematopoietic chimerism in the development of alloimmune responses[21,25,31,32]. We found an increase in the incidence and severity of GVHD and an increase in LFS in patients treated with an idarubicine-intensified conditioning regimen. GVHD is a direct reflection of increased alloreactivity, whereas LFS may at least in part reflect an increase in GVH reactivity.

We studied whether altered alloreactivity between patients treated either with or without an idarubicine-intensified conditioning regimen correlated with altered dynamics in the development of chimerism. Therefore, we randomised ten patients between recipients of allogeneic SCT that either received no intensification of the conditioning regimen, or that received intensification of the conditioning regimen with idarubicin. We increased the frequency of monitoring of chimerism, compared to the cytogenetic analysis that was performed, to 1, 3, 6, and 12 months, and annually thereafter. Moreover, we used a recently developed real-time PCR to sensitively quantify chimerism in purified subsets of PBMCs and granulocytes. The use of lineage-specific analyses further increases the sensitivity of quantitative assessment of chimerism[33]. Six patients (2 patients conditioned with idarubicine and 4 patients conditioned without idarubicine) in this group relapsed from CML. Five out of six patients responded to therapeutic DLI with a second complete remission. One patient conditioned with the intensified regimen relapsed in accelerated phase/blast crises and did not respond to DLI and died of progressive disease.

At a median of 1 month (range 1-3 months) after SCT all granulocytes and monocytes were donor derived. In the six patients who relapsed, the recipient granulocytes and monocytes started to increase at time of relapse suggesting they are part of the malignant CML clone. Recipient myeloid cells decreased in patients that responded to therapeutic DLI and became undetectable at time of second complete remission until end of follow-up. Figure 2b shows the percentages of autologous granulocytes and monocytes after SCT in UPN 480. Dynamics of persistence of autologous granulocytes and monocytes paralleled real-time RT-PCR data for Bcr-Abl and RCP as shown in Figure 2a.

Recipient NK cells defined as $CD3^-/CD56^+$ cells had completely disappeared at a median of 6 months (range 3-12) in all 10 patients. The percentage of autologous cells did not increase during relapse of CML, except in one patient (UPN 480, Figure 2c). In this patient $CD3^-/CD56^+$ recipient cells increased simultaneously with the red cells, monocytes and granulocytes during relapse of CML.

During density gradient and counterflow centrifugation not only the T cells but also B lymphocytes are depleted from the stem cell graft. This B cell-depletion results in a delayed B cell reconstitution after SCT. The first six months after SCT the number of B cells isolated by MACS and flow cytometric techniques were too small to obtain sufficient amounts of DNA for a reliable quantification by real-time PCR. Therefore, the isolation of $CD45^+/CD19^+$ cells from the peripheral blood was stopped after the fifth randomised patient.

In summary, we observed no differences in time to reach complete donor chimerism in granulocytes, monocytes and NK cells, between patients conditioned with or without idarubicine. Conversion to donor hematopoiesis was fast (within 1 month) for granulocytes and monocytes. NK conversion was complete after median 6 months.

The addition of idarubicine enhances the conversion of T lymphocytes to donor origin

T cells are important mediators and effectors of allogeneic immunoresponses. T cell chimerism was monitored in $CD3^+/CD4^+$ (Th) and $CD3^+/CD8^+$ (Tc/s) subsets (Figure 3a and b). In both groups the percentage of autologous T cells decreased after SCT and full chimerism developed in all randomised patients. In patients that received no idarubicine complete T cell chimerism developed at a median of 12 months (range 3-24) after SCT. In contrast, patients conditioned with the intensified conditioning regimen developed full T cell chimerism at a median of 3 months (range 1-12) after SCT. One month after SCT the median percentages of autologous Th and Tc cells were relatively high (median 95 and 30%, respectively) in patients conditioned without idarubicine, whereas Th and Tc cells were already lower in patients who received intensification of the conditioning regimen (median 10 and 20%). Univariate analysis showed that the addition of idarubicine contributed significantly to a lower percentage Th and Tc cells at 1 and 3 months after SCT ($p = 0.02$).

In the six patients who relapsed with CML the percentage of Th and Tc recipient cells showed a continuous decreasing pattern independently of increasing percentages of autologous myeloid and red blood cells. In figure 2a, b, and c these observations are shown for UPN 480. Patients who had not received intensification of the conditioning regimen relapsed when they still had a relatively high number of autologous lymphocytes (Figure 3a and b). Response to DLI was favourable and coincided with a rapid decrease of the percentage of autologous T cells.

These results show that intensification of the conditioning regimen with idarubicine enhances the conversion of T cells to donor origin. This enhanced hematopoietic conversion of genotype coincides with a favourable clinical outcome that may result, at least in part, from an increase in graft-versus-host (GVH) reactivity.

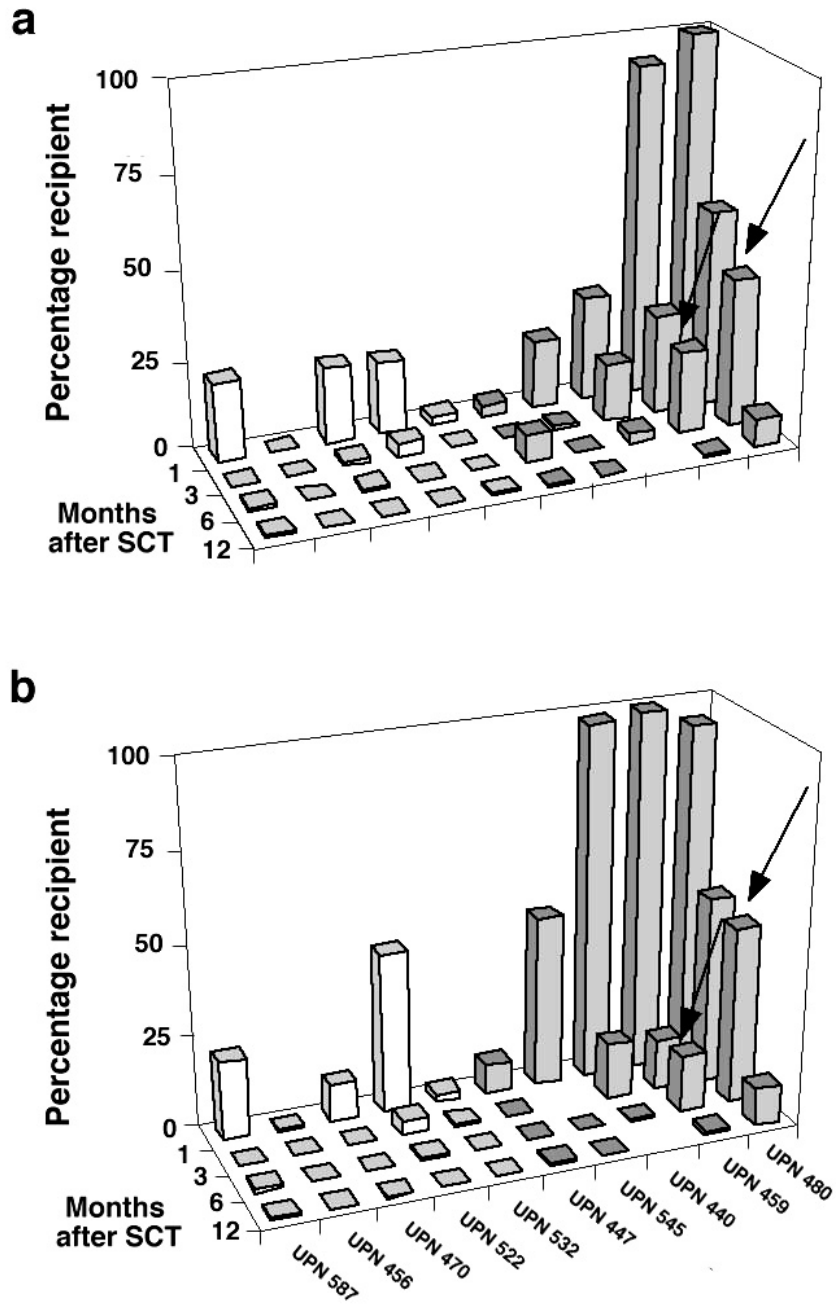

Figure 3. *Percentage autologous T lymphocytes after allogeneic SCT in CML-CP1 patients conditioned with (light bars) or without idarubicin (dark bars). (a) $CD3^+/CD4^+$ Th lymphocytes, and (b) $CD3^+/CD8^+$ Tc lymphocytes. The arrows indicate time of DLI for UPN 459 and UPN 480.*

DISCUSSION

The development of GVHD limits the success of allogeneic SCT as treatment for CML. The incidence and severity of GVHD can be reduced by removal of immunocompetent T lymphocytes from the graft. T cell depletion, however is associated with increased incidence of graft rejection and recurrent leukemia[8,10]. Addition of a fixed number of T cells to the stem cell graft after partial depletion of T lymphocytes by counterflow centrifugation, results in a low incidence of graft failure in patients transplanted for standard risk acute and chronic leukemia[22,34]. Intensification of the conditioning regimen with idarubicine reduces the incidence of relapse significantly in patients treated for AML, ALL, and CML with T cell depleted SCT[14]. Based on these results we hypothesized that the addition of idarubicine may exert an immunomodulatory effect.

In the current study we evaluated the clinical significance of an intensification of the conditioning regimen with idarubicine before allogeneic SCT in a large population of patients with Philadelphia chromosome positive CML in first chronic phase, who were either conditioned with (n = 70) or without (n = 19) idarubicine. In order to identify cellular immune mechanisms that may play a role in a differential outcome of SCT after an idarubicine intensified conditioning regimen we randomized 10 patients to receive a conditioning regimen with or without intensification with idarubicine. The choice to randomize only patients with CML was based on the knowledge that if patients would relapse, CML responds relatively well to cellular immunotherapy with donor lymphocytes.

Intensification of the conditioning resulted in a significantly lower incidence of relapse and increased LFS, concordant with earlier observations[14]. Graft failure and acute transplant-related-mortality were low. The introduction of highly sensitive molecular techniques that quantitatively detect minimal residual disease in early phase after SCT increased the amount of identified relapses. Therefore, relapse rates in this study might appear relatively high compared to studies that defined relapsed CML only as cytogenetic and/or hematological relapse. Therapeutic DLI induced complete secondary missions in the majority of relapsed patients. Therefore, the 5-year probability of current LFS was similar between both groups. Early detection of relapse may have contributed to the high number of DLI-induced secondary remissions[35]. The conditioning received before SCT did not influence response to therapeutic DLI. However, therapeutic DLI to treat relapse was administered median 25 months after SCT. This may be significantly late in relation to possible immunomodulatory effects caused by the addition of idarubicine.

The patients in this study treated with the intensified conditioning developed significantly more acute GVHD compared to the patients who received no intensification of the conditioning regimen, although clinically mild. There was a trend for more chronic GVHD in the patients conditioned with the intensified regimen. These observations are congruent with the data obtained from our previous study[14].

We considered several different mechanisms underlying the increased incidence and severity of GVHD and the lower relapse rates in patients treated with an intensified conditioning. An important factor affecting the development and severity of GVHD

may be the direct toxicity of idarubicine on tissues like gastro-intestinal mucosa, liver and skin resulting in an increased release of cytokines from these tissues with additional activation of donor T lymphocytes[18,36]. Idarubicine may directly contribute to an increased eradication of residual leukemic cells, resulting in decreased relapse rates after SCT. Furthermore, idarubicine may eliminate immunocompetent autologous T lymphocytes before SCT. In this way the potential of autologous T cells to abrogate donor T cell mediated allo-reactivity may be reduced[32,37]. Moreover, reduced HVG T cell reactivity may enhance donor T cell engraftment, and their subsequent potential to exert GVH alloreactivity.

We investigated the effect of idarubicine on the dynamics of hematopoietic chimerism as a reflection of enhanced immunoreactivity of donor derived immunocompetent cells against cells of recipient origin including the leukemic cells. Chimerism in lymphoid and myeloid subsets of 10 randomized recipients was investigated with a recently developed real-time quantitative PCR for the detection and quantification single nucleotide polymorphic alleles. The sensitivity of this technique combined with subset-specific analysis allowed accurate quantitative monitoring of hematopoietic chimerism[15,16,33]. After SCT the genotype of hematopoietic cells was frequently monitored over a period of 12 months and annually thereafter. Six patients relapsed from CML and recurrence of leukemia identified by specific detection with markers (Bcr-Abl) coincided with an increase of host hematopoiesis in myeloid subsets. In the absence of relapse we found no difference in the time to conversion to complete donor chimerism of myeloid subsets in patients with or without intensification of the conditioning regimen.

Several studies attribute important anti-leukemic activity to activated NK cells[38-41]. We did not observe a difference in hematopoietic conversion kinetics of NK cells in the patients treated with and without an intensified conditioning regimen. Six months after SCT all NK cells were donor derived. One patient (UPN 480) showed an increase in the percentage of $CD3^-/CD56^+$ (defined as NK cells) autologous cells simultaneously with the autologous red cells, monocytes and granulocytes during relapse (fig 2c). This unexpected finding may be explained by the aberrant expression of the $CD56^+$ antigen on subsets of granulocytes belonging to the CML-clone.

Autologous dendritic cells (DCs) are critical in the induction of GVHD and GVL reactivity[42,43]. We do not know whether the addition of idarubicine increased the reduction of autologous DCs. Theoretically this may result in abrogation of GVL reactivity. Host DCs, however, can prime donor T cells within 24 hours, prior to their decay as a result of the conditioning regimen[43]. Moreover, the incidence and severity of GVHD was increased after additon of idarubicine, as is the LFS, suggesting that potential enhanced reduction of autologous DC does not negatively influence the development of GVL reactivity.

A significant difference in the hematopoietic conversion from recipient to donor origin was observed in T lymphocytes. After addition of idarubicine in the conditioning regimen, both CD4- and CD8-positive subsets were almost completely donor-derived within one month after SCT. Conversion of T cells to donor origin was much slower in 3 out of 5 patients that received no intensification of the conditioning regimen. In this group 2 out of 3 patients relapsed.

These observations may confirm the hypothesis of an immunomodulatory effect mediated by idarubicine on the development of GVH reactivity after conditioning with an intensified regimen. Addition of idarubicine enhances the conversion of T cells to donor origin. Several mechanisms may contribute to enhanced conversion, including idarubicine-induced increase of 'danger' and cytoreduction of autologous T cells, resulting in enhanced engraftment of donor T cells in an environment that increases immune effector function.

Salvage therapy with DLI compensates for higher relapse rates in CML patients associated with partially T cell-depleted SCT, resulting in 5-year survival rates up to 80%[11,12,44]. We found no differences in current LFS in patients that did not receive intensification of the conditioning regimen, due to the excellent responses to DLI. DLI did largely compensate for the higher relapse rates observed in this group. However, for patients who relapse from ALL and AML the success rate of therapeutic DLI is low[30,35]. The introduction of idarubicine decreases the probability of relapse, and increases LFS, in patients transplanted for CML as well as in patients transplanted for ALL and AML[14]. Enhanced development of alloreactive responses after SCT and DLI is associated with the extent of T cell chimerism. Mixed T cell chimerism is correlated with a reduction of the development of GVH related alloreactivity, compared to full chimerism. We found a more rapid conversion of T cells to donor origin after the addition of idarubicine in the conditioning. These rapid kinetics may also explain the favourable outcome of pre-emptive DLI for patients with acute leukemia who do not suffer from significant GVHD after SCT[45], since these patients usually relapse early after SCT. Pre-emptive DLI in the context of complete donor T cell chimerism may be more immune effective compared to infusion of donor leukocytes in a situation of mixed T cell chimerism. Taken together, intensification of the conditioning regimen to prevent relapse after T cell depleted SCT of recipients that usually do not respond well to therapeutic DLI to treat relapsed leukemia, may enhance the development of GVL reactivity associated with pre-emptive DLI, because of enhanced conversion to full T cell chimerism before DLI.

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CHAPTER 7

Summary and Discussion

Allogeneic hematopoietic stem cell transplantation (SCT) following high-dose systemic chemo- or chemoradiotherapy is the most effective curative treatment for patients with leukemia. The therapeutic effect of this form of therapy is based on a steep dose-response reaction of leukemic cells to alkylating agents and/or irradiation. Unfortunately, normal tissues are also damaged, including healthy hematopoietic stem cells of the recipient. Stem cells from a donor are infused to reconstitute the hematopoietic and immune system of the recipient. Interestingly, this transplantation of donor hematopoietic stem cells provides an additional therapeutic component. Donor graft-derived T cells can mediate an immunological elimination of residual leukemic cells, called graft-versus-leukemia (GVL) reactivity. A major complication associated with donor T cell reactivity is the development of alloreactive responses towards normal host tissues, called graft-versus-host-disease (GVHD).

The conditioning treatment before allogeneic SCT suppresses the recipient's immune system and allows the engraftment and outgrowth of hematopoietic cell populations of donor origin. T cells in the graft contribute to the establishment of chimerism, that is, the engraftment and outgrowth of donor hematopoetic cells, by a graft-versus-host response towards hematopoietic cells. Patients undergoing unmanipulated allogeneic SCT usually develop full donor hematopoiesis. When the graft is T cell depleted, or when the conditioning regimen is reduced in intensity, mixed chimerism develops more frequently.

Relapse of leukemia after allogeneic SCT can be successfully treated by adoptive cellular therapy with donor lymphocyte infusions (DLI)[1-3]. Here, donor T cells are the primary therapeutic modality. However, a significant percentage of patients fails to respond to this form of therapy. In non-myeloablative allogeneic SCT, immunosuppressive conditioning is administered merely to allow donor cell engraftment, and not to eradicate the malignancy. In this way a graft-versus-host response can be generated towards a broad range of malignancies, including solid tumors. Patients treated for solid tumors with SCT after non-myeloablative conditioning or reduced intensity conditioning (RIC) only show tumor remission after full hematopoietic conversion to donor origin. Apparently, the genotype of the bone marrow, or, the extent of hematopoietic chimerism, at the time of relapse, may influence the inititation and magnitude of T cell mediated graft-versus-malignancy reactivity. Previously, we observed that a high percentage of T cells of host origin in the peripheral blood of relapsed patients at the time of DLI significantly correlates with non-responsiveness[4]. Absence of GVHD in these patients suggests that infused donor lymphocytes were reactive neither to leukemia cells nor to normal tissues. Host T cells can inhibit the development of GVHD induced by DLI in a murine bone marrow transplantation (BMT) model[5]. However, donor-derived regulatory T cells developing post-BMT may also be involved in suppression of GVHD after DLI, probably by induction of tolerance[6]. The mechanisms by which either donor-derived or recipient-derived T cells contribute to tolerance or non-responsiveness of infused donor lymphocytes have not been clearly demonstrated yet.

The research described in this thesis focusses on the role of the T cell genotype in the development of alloreactive immune responses. The first part of the thesis describes the development of a methodology to study the fate of infused T cells, and its implementation in *in vivo* mouse and rat transplantation models.

Monitoring of T cell survival, distribution and function
Survival and migration of T cells used for cellular immunotherapy are important parameters influencing treatment results. To study these parameters we chose to genetically mark T cells with a Moloney Murine Leukemia virus (MoMLV)-based vector (Chapter 2) before infusion. Real-time PCR primers and probes were designed for the detection of a MoMLV provirus-specific DNA sequence, enabling the detection and sensitive quantification of cells marked with any MoMLV-based vector. Furthermore, marked cells can be accurately quantified long-term even after *in vivo* proliferation, without loss of signal, because of stable integration of retroviral genetic sequences into the host genome. Finally, detection of cells via non-expressed genetic retroviral sequences ensures the absence of immunogenicity that may be induced by expressed marked gene sequences. This is of particular importance when studying the fate of immune effector cells in an allogeneic setting.

A prerequisite for successful retroviral transduction is that cells divide. During mitosis the nuclear membrane breaks down, allowing the reverse transcribed provirus to stably integrate into the host genome. Therefore, cells have to be stimulated *in vitro*. *Ex vivo* manipulation of cells may potentially affect their *in vivo* survival, migration patterns, and function. This could be of importance for several immunotherapeutic approaches that involve the use of *in vitro* activated T cells. CTLs used in adoptive cellular immunotherapy for EBV-induced post-transplant lymphoma and relapsed Hodgkin's disease, for example, are activated and cultured for several days to weeks to generate sufficient amounts of specific T cells. Another example is the use of T cells that have been retrovirally transduced with the HSV-Tk suicide gene, to induce GVL-activity with control of GVHD.

In Chapter 2 we showed that the period of *in vitro* stimulation and expansion of syngeneic murine splenic T cells before infusion strongly influences their survival and trafficking patterns. Cells were cultured for 3 days as the shortest culture period possible to ensure stable proviral integration and rule out discrepancies in viral DNA content per cell. After infusion a significantly higher percentage of labeled cells persisted in all tissues tested when cells had been cultured for 3 days after ConA/IL-2 stimulation in comparison to cells that had been cultured for 8 days. In addition, shortly after infusion high percentages of cells cultured for 3 days were found in lungs and liver. Thereafter, increasing percentages are found in lymph nodes and spleen. Cells cultured for 8 days preferentially migrated to liver and could hardly be detected in lymph nodes. This altered persistence and distribution of cultured cells, may reduce the alloreactive potential of these cells. Contassot *et al*., and Weijtens *et al*., described a reduced capacity of allogeneic T cells that had been cultured before infusion to induce GVHD[7,8]. Culture conditions can be adapted to restore the allogeneic effector capacity of cultured T cells in mice[7], and transduced T cells and rats[8]. Using the optimized culture and gene transduction protocol, described by Weijtens *et al*.[8], we studied the capacity of HSV-Tk transduced donor T cells to evoke GVHD in a rat model after a Class I mismatched allogeneic BMT, supplemented with retrovirally transduced T cells (Chapter 3). The *in vivo* distribution and quantity of the added

transduced T cells were related to the development of GVHD. In the peripheral blood of allogeneic transplanted rats HSV-Tk$^+$ T cells increased simultaneously with the onset and progression of GVH reactivity, and closely preceded clinical symptoms of GVHD. The percentage of allogeneic HSV-Tk$^+$ T cells was significantly higher in all tissues examined, compared to HSV-Tk$^+$ T cells in syngeneic transplanted rats, 18 days after BMT. T cells showed specific localization patterns in subepithelial regions of skin, intestines, and tongue, indicating specific homing to target sites. Also, typical GVH-induced loss of tissue architecture was observed. Notably, in GVHD target organs most T cells were HSV-Tk positive. Taken together, we showed the involvement of transduced T cells in *in vivo* alloreactivity. Furthermore, we showed that the persistence and distribution of these cells can accurately be monitored.

Reccurent T cells of recipient origin may contribute to failure of adoptive cellular immunotherapy by rejection of infused donor lymphocytes

The mechanisms by which either donor-derived or recipient-derived T cells contribute to tolerance or non-responsiveness of infused donor lymphocytes are not clearly clarified yet. In Chapter 4 we identified a mechanism that may contribute to unresponsiveness to DLI in relapsed patients. We studied the fate of infused donor T cells after DLI in recipients with hematopoiesis that reverted to host origin after allogeneic SCT. Therefore, retrovirally marked donor T cells were infused in rats repopulated with either recurrent host or persistent donor WBCs. Significant numbers of retrovirally marked donor T cells persisted after infusion in recipients that developed stable donor hematopoiesis after BMT. In contrast, infused T cells were rejected within 3 days in rats with recurrent host hematopoiesis after BMT. The rapid elimination of transferred donor T cells suggests that this clearance is an active process mediated by recipient T cells, likely sensitized *in vivo*. Recurrent T cells of recipient origin, present at the time of DLI, may therefore inhibit alloreactivity of infused donor lymphocytes by eliminating these cells. This mechanism can contribute to failure of adoptive allogeneic immunotherapy after SCT in humans who show hematopoietic cell reversion from donor to host origin.

The presence of recurrent or persisting host immunocompetent T cells after allogeneic SCT may reduce the success of subsequent adoptive immunotherapy with donor T cells. From this perspective recipient T cell immunereactivity should be suppressed to prevent abrogation of donor T cell reactivity. Moreover, T cell suppression should be confined to host T cells, which may be difficult with systemic approaches. Non recipient T cell specific immunosuppression should preferably be mild and transient.

T cell immunosuppression can be achieved via several approaches. The administration of pharmacological agents, usually used in combination (typically cyclosporin A and methotrexate), have been widely used as prophylaxis for acute GVHD (reviewed in[9]). Newer pharmacological agents, such as rapamycin[10], mycophenolate mofetil[11], trimetrexate[12], GLAT[13], and PG27[14], are under investigation. More aggressive approaches are used for the treatment of acute GVHD, such as steroids[15], and mAbs towards CD3[16] and interleukin-2 receptor[17,18]. Unfortunately, increased risk of serious infections is a major complication after high doses of steroids. Successful blocking of costimulatory molecule interactions has been achieved via systemic administration of the CTLA4-Ig fusion protein or antibodies[19], or antibodies against CD40L[20]. Van Oosterhout *et al.*[21] described the administration of immunotoxins (ITs), consisting of anti-CD3 and -CD7 mAbs conjugated to

deglycosylated ricin A (dgA) for the successful treatment of acute GVHD. T cell immunosuppression was transient and the presence of the CD3-IT provided instant immunosuppression independent of dgA-based toxicity. Moreover, treatment selectivity towards activated cells was suggested.

Increased alloimmune reactivity induced by intensification of the conditioning regimen coincides with a rapid conversion to full T cell chimerism

In the final part of this thesis we described the development of a method to quantitatively monitor the genetic origin of hematopoietic cells, and used this technique in a clinical study to monitor changes in chimerism in relation to altered alloimmune reactivity.

A major complication associated with allogeneic SCT is the development of GVHD. The occurence and severity of GVHD can be reduced by the depletion of T cells from the graft. However, the removal of immunocompetent T cells from the graft is associated with an increased risk of recurrence of leukemia. A strategy to reduce the risk of relapse after T cell depleted SCT is the intensification of the conditioning regimen. Addition of the chemotherapeutic drug idarubicine to the conditioning regimen of patients treated for CML with allogeneic SCT resulted in an increase in the occurrence and severity of GVHD and in a higher leukemia free survival (LFS) (Chapter 6). We developed allele-specific quantitative real-time PCRs for seven single nucleotide polymorphisms (SNPs), which enabled us to unilaterally discriminate 97% of HLA-identical sibling pairs. Both recipient- and donor-derived hematopoietic cells can be quantified in 67% of sibling pairs (Chapter 5). We monitored hematopoietic chimerism in different subsets after SCT, and found that the increase in GVHD and LFS after intensification of the conditioning regimen with idarubicine coincided with a faster increase in chimerism in both CD4 and CD8 T cell subsets. The addition of idarubicine to the conditioning had no differential effect on the time to reach full chimerism in non-T cell subsets. These chimerism dynamics may confirm the importance of the genotype of immune mediators in the development of allogeneic responses. Furthermore, they imply the importance of T cells in the development of allogeneic reactivity.

Monitoring of dendritic and T cell chimerism as predictive "read-out" for immune status

DCs are the most potent antigen presenting cells specialized for the initiation of primary T cell immunity[22,23]. Recent evidence in murine allogeneic BMT and DLI models demonstrated that DCs of host origin are critical in the induction of GVHD and GVL reactivity[24-26]. Frequent monitoring of chimerism, both in DCs and T cells, would provide valuable information on the immune status of the recipient before, at the time of, and after adoptive cellular immunotherapy. The assessment of DC and T cell chimerism could be used as a reliable indicator of the probable outcome of this form of therapy, and provide a platform for choosing approaches to manage adoptive immunotherapy on an individual patient basis, guiding it to a successful outcome.

We have shown that the feasibility to accurately quantify chimerism of T cells and other easily obtainable hematopoeitic cells (Chapters 5 and 6). DC chimerism, however, is difficult to monitor, since tissue DCs are difficult to obtain. The peripheral blood could provide a readily accessible source for DCs. The identification

of several DC-specific markers[27,28] has made it easier to obtain these cells from the peripheral blood, despite their scarcity.

The interrelationship, however, between the different DC subsets found in the peripheral blood that have been identified thus far, and their relation with tissue DCs, is still obscure and needs further investigation[27,28]. More importantly, little is known about their functional antigen-presenting activity, and whether this activity accurately reflects the tissue-DC function. Klangsinsirikul *et al*.[29] showed a rapid *in vivo* pretransplant reduction of circulating host myeloid ($CD11c^+$) and plasmacytoid DCs ($CD11c^-$) by the Campath-1G MoAb (anti-CD52). This may in part explain the lack of severe acute GVHD in patients after pretransplant Campath treatment[30-32], and suggest a role for circulating DCs in the overall antigen presenting-activity. However, CD52 is differentially expressed on resident DC subsets[33] and it is unclear whether in this way tissue DCs are depleted as efficiently as BDCs. In addition, donor T cell depletion at the time of marrow infusion by persistent high plasma levels of Campath-1G may also contribute to the suppression of acute GVHD[34]. Thus, it is impossible to separate the effect of T cell depletion of the graft from pretransplant host DC depletion on the development of GVHD.

Host DCs can prime naïve donor $CD8^+$ T cells within 24 hours[24,35], prior to their decay and disappearance as a result of the conditioning regime. Activated T cells can undergo as many as 7 divisions, by the time that <1% of residual host DC are detectable[24]. Thus, DCs can inititate the induction of allogeneic immune reactivity within a narrow kinetic window, and subsequently disappear, while T cells consequently expand.

In summary, both DC and T cell chimerism provide valuable information on the immunological state of 'co-existing allogeneic cells', and consequently contribute to the success of adoptive cellular immunotherapy. DC chimerism may be the most accurate prerequisite for predicting alloimmune responsiveness after cellular immunotherapy. However, tissue DCs are hard to obtain, while DCs from the peripheral blood, which are easier to obtain, may not reflect tissue DC antigen-presenting function. Ongoing research may provide answers to these questions. The role of T cell chimerism in the development of alloimmune responses is extensively studied and demonstrated. In terms of availability and quantity, T cells provide an easier, and therefore more reliable 'read-out', allowing frequent monitoring of cells with defined specificity within a dynamic process.

The adoptive transfer of allogeneic immune effector cells constitutes an exceptional setting that can be exploited therapeutically to induce responses that otherwise would be difficult to create. Better understanding of the mechanisms involved contributes to the development of means to exploit this setting.

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Samenvatting voor niet-ingewijden

Leukemie en maligne lymfomen zijn kwaadaardige aandoeningen (kanker) van het beenmerg, en organen die bloedcellen gebruiken voor hun functioneren, zoals lymfeklieren, milt, thymus, en lymfoïd weefsel in de ademhalingswegen en darmen. Bloed ontstaat uit stamcellen, die zich vermenigvuldigen en ontwikkelen tot volwassen bloedcellen, zoals witte bloedcellen (leukocyten), rode bloedcellen (erythrocyten), en bloedplaatjes (thrombocyten). Stamcellen bevinden zich in het beenmerg. Stamceltransplantatie is een effectieve manier om leukemie te behandelen. Hierbij worden kwaadaardige cellen vernietigd door middel van chemotherapie, eventueel in combinatie met bestraling van het hele lichaam. Helaas worden bij hoge behandelingdoses ook niet-kwaadaardige beenmergcellen ernstig beschadigd. De patiënt verliest daardoor zijn/haar weerstand (immuunsysteem). Infusie van stamcellen (de feitelijke stamceltransplantatie) stelt de patiënt in staat opnieuw een goed functionerend immuunsysteem op te bouwen. De in dit proefschrift beschreven stamceltransplantaties betreft transplantaties met stamcellen van een donor, de zogenaamde 'allogene' stamceltransplantie. Hierbij krijgt de patiënt als het ware het immuunsysteem van de donor (meestal een broer of zus). Dit allogene immuunsysteem blijkt in staat overgebleven (residuale) leukemische cellen, die niet door middel van chemo-/bestralingstherapie gedood konden worden, als 'gevaarlijk' te 'zien' en immuunreactiviteit te ontwikkelen tegen deze cellen. Dit noemt men *graft-versus-leukemia* reactiviteit. Het therapeutisch succes van allogene stamceltransplantaties is dus gebaseerd op enerzijds chemo- en radiotherapie, en anderzijds een immuunreactiviteit van het getransplanteerde immuunsysteem van de donor tegen leukemiecellen. Helaas beperkt het nieuwe immuunsysteem zijn reactiviteit meestal niet tot leukemische cellen, maar kan het ook een respons tegen gezonde organen van de patiënt ontwikkelen. Dit fenomeen, de zogenaamde *graft-versus-host* ziekte, is één van de grootste complicaties van allogene stamceltransplantaties.

Bij een 'standaard' allogene stamceltransplantatie worden alle bloedcellen van de patiënt vervangen door bloedcellen van donororigine. Deze patiënten zijn 'complete hematopoietische chimeren' (*full hematopoietic chimeras*). Immuunreactiviteit van donorcellen tegen residuale bloedcellen van de patiënt speelt een rol in het 'aanslaan' en uitgroeien van deze cellen. Als van het 'standaard' protocol wordt afgeweken, kan een situatie ontstaan waarbij immuunsystemen van donor en ontvanger origine naast elkaar bestaan. Dit noemt men 'gemengd chimerisme' (*mixed hematopoietic chimerism*). Denk hierbij bijvoorbeeld aan een voorbehandeling van de patiënt met lagere doses chemotherapie, waardoor het immuunsysteem van de patiënt minder verzwakt is, en het als het ware weerstand kan bieden aan de immuunreactiviteit van het transplantaat. Het naast elkaar bestaan van twee immuunsystemen, die in principe in staat zijn om een respons tegen elkaar te ontwikkelen, betekent dat er sprake is van regulatie van immuunreactiviteit. De manier waarop immuunresponsen worden gereguleerd is zeer complex.

T cellen zijn witte bloedcellen die een belangrijke rol spelen bij de ontwikkeling en, met name, de uitvoering van allogene responsen. Zij kunnen er echter ook voor zorgen dat er juist geen immuunrespons optreedt, de zogenaamde tolerantie-inductie.

T cellen kunnen als zogenaamde 'effectorcellen' gebruikt worden als therapie. Dit gebeurt met name ná allogene stamceltransplantatie, wanneer de leukemie 'terugkomt' (recidiveert). Meestal gaat het hierbij om residuale leukemiecellen die chemotherapie, radiotherapie, én donor immuunreactiviteit hebben overleefd, en weer gaan woekeren. Infusie van donor T cellen van dezelfde donor als van de stamceltransplantatie, kan leukemiecellen opruimen, door *graft-versus-leukemia* reactiviteit. Met name gerecidiveerde chronische myeloïde leukemie is op deze manier goed te behandelen.

De ontwikkeling van immuunresponsen is een complex proces, dat door een groot aantal factoren wordt beïnvloed. In dit proefschrift wordt de rol van T cel chimerisme in de ontwikkeling van *graft-versus-leukemia* en *graft-versus-host* reactiviteit beschreven, en één van de mechanismen die mogelijk een rol spelen in verminderde immuunreactiviteit na therapie met donor T cellen. Om dit te kunnen onderzoeken hebben we een techniek ontwikkeld om T cellen na infusie in het lichaam te kunnen vervolgen (Hoofdstuk 2). Bij sommige vormen van immuuntherapie met T cellen is het noodzakelijk om deze cellen tot grotere hoeveelheden op te kweken vóórdat ze getransplanteerd worden. In Hoofdstuk 2 is tevens beschreven dat dit een nadelig gevolg kan hebben voor de therapeutische werking van deze cellen, omdat ze eerder uit het lichaam van de ontvanger kunnen verdwijnen. Het opkweken van deze cellen kan op een zodanige manier worden aangepast dat ze niet meer sneller verdwijnen dan geïnfundeerde 'verse' T cellen.

Hoofdstuk 3 laat zien dat het vermeerderen van T cellen in het laboratorium vóór transplantatie geen invloed heeft op het functioneren van deze cellen, en dat ze een immuunrespons kunnen bewerkstelligen vergelijkbaar met 'verse' T cellen. In het onderzoek dat in dit hoofdstuk is beschreven hebben ratten een beenmergtransplantatie gekregen met gekweekte T cellen daaraan toegevoegd. Gebruik makend van, onder andere, de techniek die in Hoofdstuk 2 is beschreven, laten we zien dat de ratten ernstige *graft-versus-host* ziekte krijgen, en dat de gekweekte T cellen daar mede voor verantwoordelijk zijn. Dit blijkt onder andere uit het feit dat de T cellen zich sterk vermenigvuldigden rond de tijd dat de ratten ziek werden. Daarnaast bevonden de T cellen zich met name in organen die sterk aangedaan waren door de *graft-versus-host* ziekte.

In Hoofdstuk 4 hebben we in ratten een situatie nagebootst die zich voordoet bij patiënten waarbij de leukemie terugkomt: patiënten worden behandeld met een stamceltransplantatie, waarna hun bloedcellen worden vervangen door bloedcellen afkomstig van de donor. Als de residuale leukemische cellen gaan uitgroeien kan dit gepaard gaan met een terugkeer van bloedcellen van patiënt origine. Wanneer men deze patiënten vervolgens behandelt met donor T cellen kan het ontwikkelen van *graft-versus-leukemia* activiteit uitblijven. Ook *graft-versus-host* ziekte komt minder voor. Blijkbaar kunnen de geïnfundeerde donor T cellen hun werk in deze patiënten minder goed uitvoeren. In ons rattenmodel hebben we laten zien dat dit komt, doordat donor T cellen na infusie sneller verdwijnen in ratten die hun eigen bloedcellen hebben teruggekregen, dan in ratten die bloedcellen van de donor hebben. Waarschijnlijk komt dit doordat 'patiënt' T cellen de donor T cellen opruimen.
Bij een allogene transplantatie is de donor vaak een broer of zus van de patiënt. Dat betekent dat ze genetisch gezien erg op elkaar lijken. Dat is voordelig, want het bepaalt mede de geschiktheid van de stamcellen voor transplantatie. Het maakt het

echter voor onderzoekers moeilijker om een onderscheid te kunnen maken tussen bloedcellen van de patiënt en donor na een allogene stamceltransplantatie. In Hoofdstuk 5 is een techniek beschreven waarmee onderscheid gemaakt kan worden tussen donor- en patiënt-cellen op basis van minieme genetische verschillen, de zogenaamde *single nucleotide polymorphisms*. Hiermee kan de mate van chimerisme in een patiënt na transplantatie worden bepaald. Dit is belangrijk omdat hierdoor, bijvoorbeeld, transplantaatafstoting voorspeld kan worden of het gaan uitgroeien van residuale leukemische cellen. Daarnaast kan het belangrijke informatie geven over het al dan niet ontwikkelen van imuunresponsen van donor cellen tegen de ontvanger.

Hoofdstuk 6 beschrijft een klinisch onderzoek, waarin een groot aantal patiënten voorafgaand aan allogene stamceltransplantatie is behandeld met het chemotherapeutisch medicijn idarubicine. Deze groep is vergeleken met patiënten die de 'standaard' behandeling voor stamceltransplantatie hadden ontvangen. Patiënten behandeld met idarubicine bleken een lagere kans op een terugkeer van de leukemie te hebben, mogelijk als gevolg van een hogere *graft-versus-leukemia* reactiviteit. Daarnaast ontwikkelden zij een hogere, zij het klinisch milde, mate van acute *graft-versus-host* ziekte. Gebruik makend van de techniek beschreven in Hoofdstuk 5 hebben we laten zien dat in patiënten behandeld met idarubicine de T cellen van patiënt origine sneller werden vervangen door T cellen van donor origine, dan in de patiënten die niet met idarubicine waren behandeld. Hierdoor is de relatie anti-patiënt-immuunreactiviteit met donor T cellen, na manipulatie met idarubicine, aangetoond.

Samenvattend kunnen we stellen dat de aanwezigheid van T cellen van patient origine de activiteit van donor T cellen, dus ook de therapeutisch gunstige *graft-versus-leukemia* reactiviteit, kan verminderen. De negatieve werking van autologe T cellen zou op een zodanige manier gemanipuleerd kunnen worden dat er een optimaal rendement uit therapie met donor T cellen gehaald wordt. Dit kan bijvoorbeeld door de reactiviteit van patient T cellen (tijdelijk) te onderdrukken. Het is hiervoor belangrijk om het genotype van T cellen na stamceltransplantatie, en in aanloop van, en na immuuntherapie met donor T cellen, te (kunnen) volgen.

De infusie van donor T cellen biedt een uitzonderlijke basis die kan worden benut voor therapeutische doeleinden die op een andere manier moeilijk te realiseren zouden zijn. Beter begrip van de mechanismen die hierbij betrokken zijn draagt bij aan de ontwikkeling van methoden om deze vorm van therapie uit te buiten.

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Curriculum Vitae

De schrijver van dit proefschrift werd geboren op 16 april 1970, te Eindhoven. In 1988 behaalde hij het Atheneum-B diploma aan het Lorentz Lyceum te Eindhoven, waarna hij begon met de studie Biologie aan de Katholieke Universiteit Nijmegen. In het kader van deze studie werden stages gelopen op de afdeling Hematologie (Prof. Dr. T. de Witte) van het UMC St Radboud en de afdeling Moleculaire Plantenfysiologie (Prof. Dr. G. Wullems) van de Katholieke Universiteit Nijmegen. In augustus 1994 behaalde hij het doctoraal diploma Biologie met als specialisatie 'Medische Biologie'. Vanaf begin 1995 heeft hij als vrijwilliger onderzoek verricht op het laboratorium voor Biochemie (Dr. W. de Grip). In april 1996 is hij als assistent in opleiding aan een promotieonderzoek op de afdeling Hematologie van het Universitair Medisch Centrum (UMC) Utrecht (Prof. Dr. J. Sixma, Prof. Dr. H. Clevers) begonnen. In mei 1998 is dit project beëindigd, waarna in juni 1998 is begonnen aan een promotieonderzoek op de afdeling Hematologie van het UMC St Radboud te Nijmegen (promotor: Prof Dr. T. de Witte, en co-promotores: Dr. E. van de Wiel-van Kemenade en Dr. H. Dolstra). De resultaten van dit onderzoek zijn in dit proefschrift beschreven.

De schrijver woont samen met Birgit Vullinghs en is de trotse vader van Iris.

‘I wish I was as fortunate,
as fortunate as me’
- Eddie Vedder, *Wishlist*, ***Yield*** (1997)

Erratum

Some of the primer sequences have not been correctly displayed in Table 2 (page 93) and Table 1 (appendix; page 107) of Chapter 5. The following Table shows the correct primer and probe sequences for all PCRs described in the chapter.

Gene/STS	SNP	Allele-specific primer (5'-3')[a]	Common primer (5'-3')	Probe (TET-5'-3'-TAMRA)
PECAM1	C	AGGACTCACCTTCCACCAAC**C**<u>**G**</u> (R)	GGATCTATGACTCAGGGACATATAAATG (F)	TGTGAACAACAAAGAGAAAACCACTGCAGAGT
	G	AGGACTCACCTTCCACCAAC<u>**T**</u>**C** (R)		
ICAM1	G	AGAGCACATTCACGGTCACC<u>**A**</u>**C** (R)	GCACTTTCCCACTGCCCAT (F)	CAGTGACTGTCACTCGAGATCTTGAGGGC
	A	AGAGCACATTCACGGTCAC<u>A</u>T**T** (R)		
HA1	G-T	GCTCTCACCGTCA**C**GCAA (R)	TGCTGGCGGACGTGG (F)	CCGCTTCGCTGAGGGCCTTGA
	A-C	GGCTCTCACCGTCA**T**GC<u>**C**</u>**G** (R)		
MLH1	G	TCGTGCTCACGTTCTTCC<u>T</u>C**C** (R)	GAGACCCAGCAACCCACAGA (F)	ATTCAAGCTGTCCAATCAATAGCTGCCG
	A	TCGTGCTCACGTTCTTCC<u>A</u>T**T** (R)		
SUR1	C	TGCCACCCTCCCTCCCT<u>A</u>**C** (F)	GACAGCCCCTGAGACCTTCTG (R)	CAGGTGGGCTGCGGCAAGTCC
	T	TGCCACCCTCCCTCCCT<u>A</u>**T** (F)		
G42863[b]	C	GGCTTGTGGATGAAGGAG<u>T</u>**C** (F)	TGGCACATCTGGCAAAATCTC (R)	ACAGGGAGAGTGATGTTGGAGCTGGGT
	A	GGCTTGTGGATGAAGGAG<u>A</u>**A** (F)		
G42888[b]	C	GGGGAGGGGAGGAAGAGA<u>G</u>**C** (F)	TTGTGCTGCTGTAATCACTTTTCAT (R)	TTCTCTCTCGGGATTTTCTGGGAATCAAAA
	T	GGGGAGGGGAGGAAGAGA<u>C</u>**T** (F)		
SMCY[c]	n.a.	TCTTGCGTCCTCAGCGTTTA (F)	AGTGTGGTACGAGCCGTCTCA (R)	CTCAGGTGCGGAAGGTCTCACAGGTT

[a] Polymorphic nucleotides are given in bold, and introduced mismatched nucleotides to decrease background amplification are underlined.
[b] Sequence-tagged site.
[c] Male specific PCR.
TET, tetrachloro-6-carboxy-fluorescin; TAMRA, 6-carboxy-tetramethyl-rhodamine; F, forward primer; R, reverse primer; n.a., not applicable.